REPRODUCTIVE BIOLOGY AND POPULATION MOLECULAR GENETICS OF THE SCRUB MORNING GLORY *BONAMIA GRANDIFLORA*

By

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by

Gabriela B. Romano

I dedicate this work to my parents, Beatriz Carballo and Aníbal Romano, my husband, Don A Baldwin, and my sister, Marcela Romano.

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Abstract of Dissertation Presented to the Graduate School
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REPRODUCTIVE BIOLOGY AND POPULATION MOLECULAR GENETICS
OF THE SCRUB MORNING GLORY *BONAMIA GRANDIFLORA*

By

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Chairman: David A. Jones
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The Florida scrub is an ancient ecosystem that appeared twenty million years ago and has persisted to the present. *Bonamia grandiflora,* the scrub morning glory, is one of the many threatened or endangered species endemic to the Florida scrub. This study assessed the phenotypic variation, reproductive biology and molecular genetic diversity of *B. grandiflora* throughout its geographic range.

Variation in morphology was assessed in wild populations of B. *grandiflora.* The number of stems per plant and stem length were variable between populations; the reproductive output, leaf pubescence (number of hairs/mm^2) and flower dimensions were not. There was no geographic pattern associated with any of the variables. Variation in number and length of stems may be due to habitat differences. Although reproductive

output is associated with the number and length of stems, other unknown factors have as much influence as plant size.

The breeding system of *B. grandiflora* was investigated through hand-pollinations in three populations in the Ocala National Forest. *B. grandiflora* has a mixed mating system; that is, its seeds form both by selfing and by cross-pollination of the flowers. Natural pollinators are necessary to ensure substantial seed production. *B. grandiflora* shows some inbreeding depression in selfed fruits and seeds but this does not appear to hinder the present populations; however, germination percentages are generally low. Seed herbivory rarely occurs and does not endanger this species.

The genetic diversity in *B. grandiflora* was assessed by isozymes, RAPD markers and AFLP markers. Genetic variation in this morning glory is reduced and comparable to near-isogenic agronomic lines and some very rare species. The genetic diversity is allocated mainly within rather than between populations. This results in poor structure of the genetic richness and a very weak geographic pattern of this diversity. Two populations have the greatest genetic diversity but no single population covers the entire gamut of genetic variation.

B. grandiflora does not appear in immediate danger of extinction but it has poor genetic diversity and it shows some inbreeding depression. Further reduction of its natural populations could exacerbate these problems and impede the long-term survival of the scrub morning glory.

CHAPTER 1
GENERAL INTRODUCTION

Bonamia grandiflora (Gray) Heller is one of the many threatened or endangered species endemic to the Florida scrub (Ward, 1979). Austin (Austin 1979) remarks that 'the largest number of surviving plants of *Bonamia grandiflora* is in the Ocala National Forest'. He goes on to explain that forest practice of close replanting and the exclusion of fire are largely responsible for the disappearance of the species from that habitat.

The USDA Forest Service, mindful of the problem, proposed the reintroduction of the species to a number of sites in the forest. We were asked whether it would be important to consider the provenance of the seed for such a program. The rationale was that if there were no genetic variation between seeds of different origin, then the source of the seed would be unimportant. On the other hand, if there were much variation between populations or sub-populations of *B. grandiflora* over its range, then careful choice of seed would be necessary. A field trial had already been established at the Seed Orchard of the Ocala National Forest, a few miles north of the known natural distribution of the species and so some source material was readily available.

This study assessed the phenotypic variation and genetic diversity in several populations of *B. grandiflora* throughout its geographic range and its reproductive biology in populations in the Ocala National Forest.

The genus *Bonamia* belongs to the Convolvulaceae, a family characterized by herbaceous and shrub species, many of them vines, with bell-shaped flowers. These plants usually have toxic milky latex and several species, such as sweet potato (*Ipomoea batatas*), have underground storage organs with anomalous secondary growth (Metcalfe and Chalk, 1950). *Bonamia* has an underground storage organ but it is woodier than that of the sweet potato (Myint and Ward, 1968). *Bonamia* is a relatively large genus compared with others in the Convolvulaceae. Forty-six species have been described (Myint and Ward, 1968). It occurs in tropical and subtropical areas of the Northern and Southern Hemispheres; in North, Central and South America, South Africa, Australia, Hawaii, and tropical Asia (Myint and Ward, 1968). In contrast to the widespread distribution of the genus, only five of the forty-six species have large geographic distributions; the rest have limited distributions and rare occurrences (Austin, 1994; Myint and Ward, 1968). Many *Bonamia* species live in sandy soils, some on coastal plains, open forests, scrubby lands, edges of forests, or along streams, and a few of them have unknown habitats and are only known from their type specimens (Myint, 1968; Myint and Ward, 1968).

Until Myint and Ward's revision of the genus *Bonamia* (1968), species that now belong to *Bonamia* were included in the genera *Stylisma*, *Breweria*, *Breweriopsis*, *Trichantha*, and *Perispermum*. The reverse is true for species of *Stylisma*, which used to be in *Bonamia* (Myint and Ward, 1968). It appears that the only consistent difference between all the species in *Bonamia* and all the species in *Stylisma* is that in the former genus the cotyledons are oval, oboval or bilobed while in the latter the cotyledons are bifurcated (Myint and Ward, 1968). A later study of pollen grain anatomy by scanning

electron microscopy found that in *Stylisma* species pollen grains have an advanced 3-aggrecolpate aperture. All American and Australian species of *Bonamia* have a primitive 3-colpate aperture although *B. thunbergiana* (African) does have the advanced 3-aggrecolpate aperture (Lewis, 1971).

The species *Bonamia grandiflora* is a perennial, trailing vine with a somewhat woody underground storage organ from which one to many above ground stems arise. *B. grandiflora* has no tendrils, thorns or other organs to aid in climbing and the stems do not twine around supports, they are prostrate on the ground. The leaves are sessile or subsessile, subcoriaceous and ovate (2 to 3 cm long, 2 to 2.5 cm wide) and the flowers are axillary, solitary, deep blue to purplish-blue (7 to 8.5 cm long, 5 to 7 cm wide) (Myint and Ward, 1968). The fruits are four-seeded capsules, typical of the family, and the seeds are glabrous, oval and brownish (Myint and Ward, 1968). The above ground stems die back during winter and new stems grow out of the underground storage organ. No adventitious roots arising from the above ground stem have been reported; this species does not propagate asexually under natural conditions. Although the identification of young individuals may be difficult if several are growing in close proximity, as the plants grow older and produce more stems, the delimitation of individuals becomes easier. Is possible that some self-thinning occurs in stands of *B. grandiflora* since large plants have not been observed growing near other individuals.

B. grandiflora is geographically isolated from other *Bonamia* species that occur in Mexico and Central America (Myint and Ward, 1968). This species was listed as threatened on November 2, 1987, in the Federal Register. *B. grandiflora* has been reported as extirpated in Sarasota, Manatee, Osceola, Volusia, and Lake Co. and present

but reduced in Marion, Hardee, Highlands, Orange and Polk (U.S. Fish and Wildlife Service, 1987). However, a couple of populations in Lake County were located and sampled in this study.

B. grandiflora typically grows on white sands together with *Ceratiola ericoides* (rosemary), *Pinus clausa* (sand pine), *Serenoa repens* (saw palmetto) *Lyonia ferruginea* (rusty lyonia), and oaks, *Quercus myrtifolia* (myrtle oak), *Q. geminata* (sand live oak), *Q. chapmanii* (Chapman's oak) among other species. All of these species have been associated through the recent geologic history of the Florida scrub. In the Ocala National Forest *B. grandiflora* has been found in longleaf pine flatwoods (personal observation) and in the Tiger Creek Preserve in Polk County it is found in sandhills, associated with *Quercus laevis* (D. Gordon personal communication).

The Florida peninsula emerged during the late Oligocene and it appears that, more than twenty-five million years ago, the first terrestrial vegetation type in Florida was a mixed hardwood forest (Webb, 1990). Sandhills and scrubs appeared approximately five million years after the hardwood forests, during the early Miocene (Webb, 1990). Towards the end of the Miocene the global water level dropped even more and savannas became more widespread (Webb, 1990). During the Pliocene (Cenozoic) there was a rich semiarid biota with numerous elements in common with the dry areas in the western U.S. (Webb, 1990). At the end of the Pliocene, scrub habitats were widespread in Florida, as indicated by the presence of *Selaginella arenicola* (spike moss), *Serenoa*, *Ceratiolla* and *Polygonella* (Watts, 1975), and there was a corridor to the southwestern U.S. around the Gulf Coast (Webb, 1990). This was followed by a relatively warm period and later by the Wisconsinan glaciation (Watts, 1975).

During the Holocene there were alternations of drier and wetter periods due to changes in rainfall, water tables, or both. At present the Florida peninsula is wetter than when scrub habitats were widespread (Watts, 1975).

The scrub that once extended from coast to coast through a much wider Florida peninsula is now circumscribed to islands that sit on the Florida Central Ridge, the Big Scrub (Ocala) and the Lake Wales Ridge. There are also several smaller patches of coastal scrub on the Gulf and Atlantic coasts. A large part of the Big Scrub is within the Ocala National Forest (Myers, 1990). The Lake Wales Ridge scrub patches have lost more than 70% of their area to citrus cultivation and development (Peroni and Abrahamson, 1985) and much of the rest of the Central Ridge has had similar losses (Myers, 1990).

There is a group of scrub plant and animal species with southwestern U. S. affinities; these species entered the peninsula during the Pliocene. The plant species are *Palafoxia feayii* (palafoxia), *Ziziphus celata* (Garret's ziziphus), *Carya floridana* (scrub hickory), *Liatris ohlingerae* (Florida gayfeather) and *B. grandiflora* (scrub morning glory) (Huck *et al.*, 1989). The animals are, among others, several lizards, snakes and skinks, *Cnemidophorus*, *Eumeces, Lampropeltis, Micrurus, Pituophis, Cemophora*, *Sceloporus*, and the bird *Aphelocoma coerulescens* (Florida scrub jay) (Webb, 1990). If all these organisms migrated from the SW U.S. or even from Mexico or South America, Florida represents the extreme eastern end of their geographic distribution. Populations in Florida would be far from the center of origin or the central populations and so impoverished gene pools can be expected in these species (Playford *et al.,* 1993; Boursot, *et al.,* 1996; Hafner and Schuster, 1996; Villand *et al.,* 1998).

To the supposedly already poor genetic diversity due to being at the edge of their distribution, we must add the effect of habitat fragmentation following the drastic reduction in area during the Holocene. It is generally accepted that habitat fragmentation can generate genetic fragmentation of the original gene pool into separated units (Templeton *et al.*, 1990). The particular effects depend on demographic as well as on historical factors (Templeton *et al.*, 1990). If the original population is highly polymorphic, and the remaining subpopulations are large enough to continue existing as evolutionary units, no significant genetic loss should occur. If the subpopulations are not quite as large but there is gene flow between them, then there would not be overall genetic impoverishment. Over time, we could expect clines according to the effective distance of gene flow. For example, in the scrub jay (*Aphelocoma coerulescens*), there were genetic similarities between the Ocala and the Atlantic coastal scrub populations and between those of the Lake Wales Ridge and the Gulf coastal scrub (Scott Steward, personal communication).

If the initial populations were fragmented into very small subpopulations, without any possibility of gene flow between them, genetic drift would be likely and ultimately extinction could occur (Templeton *et al.*, 1990). Even if genetic drift did not occur, demographic factors are bound to have a negative effect on these small subpopulations through the Allee effect (Lande, 1988). In animals it has been shown that, in small and sparse populations, social interactions necessary for reproduction may be hindered or the probabilities of finding a mate reduced (Allee *et al.*, 1949). In the case of plant species, reduced attractiveness to pollinators and isolation from other individuals and therefore reduced pollination efficiency are likely to happen in small and isolated populations.

To facilitate the management of rare species through their reintroduction in areas from which they have been lost due to human activities or by artificially increasing gene flow, it is advisable to have good knowledge of their natural history, their demography, their genetic structure, their breeding system in the case of plant species, and their behavior in the case of animal species.

Before relocating plant species as mature individuals, seeds or pollen, the genetic structure of the natural populations should be assessed. Knowledge of the genetic diversity of a species and the allocation of this diversity is necessary to decide which populations, if any, appear more valuable for conservation purposes. This knowledge may also prevent the accidental mixture of different gene pools, which could affect the integrity of the species by disrupting genotypes that may have local adaptations or have an overall homogenizing effect in those gene pools or both (Chapin and Chapin, 1981; McGraw and Antonovics, 1983; Schmidt and Levin,1985; Templeton , 1986; Fischer and Matthies, 1997).

In order to decide whether urgent intervention is required to ensure the survival of rare plant species that are impacted by habitat loss and fragmentation, it is necessary to assess whether inbreeding depression is affecting their populations. This is particularly important if the genetic diversity of those species appears to be reduced, increasing the probability of inbreeding depression due to an increase in the frequency of homozygous individuals.

Characterization of the mating system is also relevant for plant species management because isolation of small populations will have different impacts on primarily selfing and primarily outcrossing species. Small populations from primarily

selfing species may show little impact, at least in the short term, since they do not require other individuals to reproduce successfully. Primarily outcrossing species need a larger number mature individuals in order to have reproductive success. The degree of inbreeding depression experienced by species also depends on the breeding system. Species that are mainly selfers have gradually eliminated numerous lethal and semilethal mutations from the gene pool and so inbreeding depression becomes less likely to occur (Charlesworth and Charlesworth, 1987).

The objective of this study was to address some of these biological aspects that will have direct bearing on the conservation of *B. grandiflora*. This study set out to analyze the phenotypic and genotypic variation of this species as well as its reproductive biology. The plants growing in the Ocala Seed Orchard in a common garden arrangement exhibited some phenotypic variation. To try to verify phenotypic variability, attempts were made to establish plants from nine *B. grandiflora* populations in the greenhouse from seed and by tissue culture techniques. Because these experiments were unsuccessful (and will not be reported here) observations and experiments were carried out on plants in their natural habitats.

Several populations of *B. grandiflora* distributed throughout its geographic range were assessed for phenotypic variation by measuring ten morphological characters. The mating system of *B. grandiflora* was investigated through experimental hand pollinations; subsequently the resulting fruits and seeds were used to assess whether this species showed inbreeding depression. The genetic diversity in *B. grandiflora* was investigated through enzyme electrophoresis, RAPD and AFLP marker analysis. Where relevant, the data were analyzed by suitable statistical methods. Finally, all of the

information gathered was used to make recommendations for the management of this rare scrub endemic.

CHAPTER 2
PHENOTYPIC CHARACTERIZATION OF POPULATIONS OF *BONAMIA GRANDIFLORA*

Introduction

Previous research on *Bonamia grandiflora* includes a taxonomic study (Myint and Ward, 1968), records of its distribution in the Florida scrub (Florida Natural Areas Inventory, Tallahasee, FL) contributed mainly by S. P. Christman (Christman 1988), and a study of the effects of fire on mature plants and on seed bank dynamics (Hartnett and Richardson, 1989).

As it was mentioned in Chapter 1, the plants growing in the Ocala National Forest Seed Orchard in a common garden arrangement exhibited some morphological variation. Therefore, attempts were made to establish a common garden with plants from throughout the geographic range of *B. grandiflora* both from seed and through tissue culture. These experiments were unsuccessful so morphological variables were quantified in natural populations.

This study assessed the status, size and reproductive potential, of individuals in several populations of *B. grandiflora.* The results can be used as a reference in future assessments of those populations to determine whether the populations appear stable or in decline.

The present project had the objective of characterizing *Bonamia grandiflora* populations with respect to several phenotypic characters that are relevant to

reproductive success. These characters are plant size; reproductive output; seed weight, size and allometry; and percentage germination. Other morphological characters measured were leaf pubescence and flower dimensions.

B. grandiflora leaves have been described as puberulous or glabrescent (Myint and Ward, 1968). Increased reflectance of sunlight can be measured in white, pubescent leaves (Gates and Papain, 1971). However, *B. grandiflora* leaves do not look grayish or glaucus and so their pubescence does not seem to be reflecting sufficient light to have an impact on leaf temperatures and therefore to confer any selective advantage. Pubescence may also help to reduce evapotranspiration by increasing the boundary layer around the surfaces of the leaves.

Bonamia grandiflora does not appear to have specialized pollinators. Its large flowers are visited by a variety of insects, ranging from small colletid bees to large swallowtail butterflies. Therefore, small variations in flower dimensions are unlikely to decrease seed production due to lack of pollination. If there were genetic variation in this species affecting corolla morphology it is expected that flower dimensions will be different between populations.

Methods

To characterize the populations and assess whether morphological differences between populations exist various subsets of twelve populations of *B. grandiflora* were sampled. These populations were located throughout the geographic range of this species (Fig. 2-1).

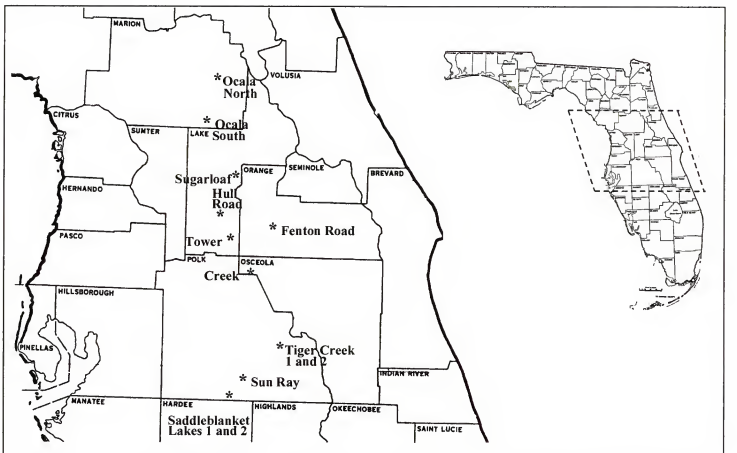


Figure 2-1. Populations of *Bonamia grandiflora* that were sampled in the phenotypic characterization of this species. All of them are located on the Central Ridge of the Florida peninsula.

Morphometric data were recorded for twenty individuals randomly chosen in nine of the twelve populations. The following data were collected for each plant: number of stems, length of each stem, reproductive output as measured by the number of capsules, flowers and flower buds produced by each meter of stem. The stems were measured from the point at which they emerge from the ground. To ensure that the data would be comparable, all the morphometric measurements were done during the same month.

Seed weight was measured in eight of the twelve populations with a Mettler analytical balance. The seeds were collected at random from the field and kept with a desiccant for several months until their weight was measured. No more than four seeds (the contents of a capsule) were collected per plant. In some cases, seeds could only be found individually on the ground by the parent plants, and again only four seeds were collected for each individual. Seed dimensions were measured in five populations by a Spi vernier caliper. The ratio of seed length divided by its width was calculated for each individual seed.

The germination of seeds obtained at random from four of the twelve populations of *B. grandiflora* in a mixture (1:1) of commercial sand and sand collected in the Ocala National Forest scrub was estimated. To allow the imbibition of the seeds, the seed coats were sanded (scarified) and the seeds were covered by approximately 0.5 cm of moist sand. All the seeds in a capsule, typically four, constituted a replicate.

Leaf pubescence (number of hairs/mm^2) was quantified in the abaxial and adaxial surfaces of ten leaves in four of the twelve populations. The leaves were examined with the aid of a dissection microscope. Two of the populations were at the southernmost and

northernmost extremes of the geographic range of *B. grandiflora* and the other two were from the central area of the distribution.

Corolla length, diameter and the depth of the cleft in the corolla in 30 flowers in three of the twelve populations were measured by a Spi vernier caliper. Only one flower was measured for each plant and they were chosen at random. To ensure that the data would be comparable, these morphometric measurements were done during the same month.

The number of stems/plant, length of stems, seed length, seed width, the ratio of seed length and width, leaf pubescence and flower dimension were analyzed by ANOVA. A completely randomized design was used in all the analyses. The number of stems/plant and length of stems were transformed (logarithmic transformation) to meet ANOVA assumptions. Duncan Multiple Comparisons were used as follow-up tests for significant ANOVAs. Transformations did not achieve normality of residuals or homoscedasticity in reproductive output and percentage germination, so a Kruskal-Wallis non-parametric test was used (Sokal and Rohlf, 1981).

Results

The number of stems/plant was variable between populations (ANOVA, $F = 9.56$, $df = 8$ and 171, $P < 0.05$, Table 2-1). A Duncan multiple-range test showed that the two populations in the Tiger Creek Preserve had the fewest stems, and one of the Saddleblanket populations had the most numerous stems ($\alpha = 0.05$; Fig. 2-2). The other populations had intermediate stem lengths and numbers (Fig. 2-2).

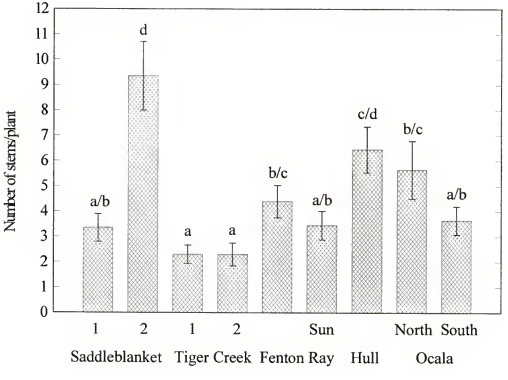


Figure 2-2. Number of stems per plant (mean ± s.e.) in nine populations of *Bonamia grandiflora* in the Florida scrub. Populations with different letters are significantly different (Duncan multiple-range, $\alpha = 0.05$).

Table 2-1. ANOVA of the number of stems/plant in nine populations of *Bonamia grandiflora* in the Florida scrub.

Source	df	SS	MS	F	P
Between populations	8	6.81	0.85	9.56	< 0.05
Within populations	171	15.23	0.09		
Total	179	22.04			

The length of stems was also variable between populations ($F = 23.45$, $df = 8$ and 171, $P < 0.05$, Table 2-2). Parallel to the results of number of stems/plant, a Duncan multiple-range test showed that the two populations in the Tiger Creek Preserve had the shortest stems, and one of the Saddleblanket populations had the longest stems ($\alpha = 0.05$; Fig. 2-2 and 2-3). The other populations had intermediate stem lengths and numbers (Fig. 2-3).

Table 2-2. ANOVA of the stem length (m) in nine populations of *Bonamia grandiflora* in the Florida scrub.

Source	df	SS	MS	F	P
Between populations	8	18.58	2.32	23.45	< 0.05
Within populations	171	16.93	0.10		
Total	179	35.51			

Reproductive output, the sum of the number of capsules, flowers, and flower buds, was not different between populations (Kruskal-Wallis, $H = 10.80$, $\alpha = 0.05$; Fig. 2-4). However, there was a strong trend for the Sun Ray populations to produce the largest number of capsules, flowers, and flower buds per meter of stem (Fig. 2-4).

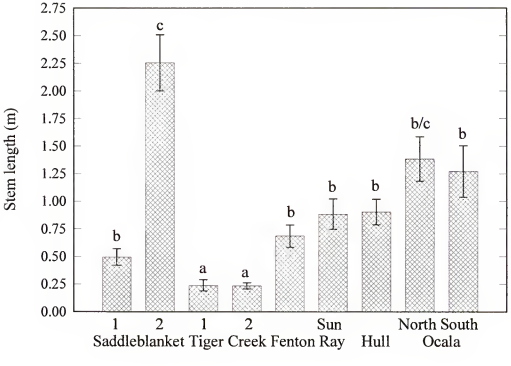


Figure 2-3. Stem length (mean ± s.e.) in nine populations of *Bonamia grandiflora* in the Florida scrub. Populations with different letters are significantly different (Duncan multiple-range, $\alpha = 0.05$).

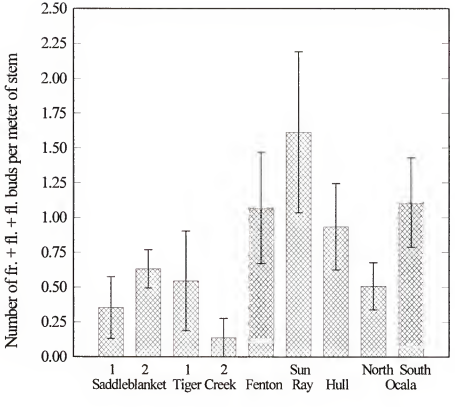


Figure 2-4. Reproductive output, number of fruits, flowers and flower buds (mean ± s.e.) in nine populations of *Bonamia grandiflora* in the Florida scrub.

The reproductive output was associated with total stem length ($r^2 = 0.53$, df = 181, $P < 0.05$). When only the number of capsules was used in the regression, the association with the total length of stems was similar ($r^2 = 0.51$, df = 181, $P < 0.05$).

Seed weight was different between populations ($F = 21.28$, df = 7 and 424, $P < 0.05$; Table 2.-3). Tower produced the lightest seeds and Sun Ray, the heaviest, followed by Fenton and Saddleblanket (Duncan multiple-range test, $\alpha = 0.05$; Fig. 2-5). The other populations had intermediate average weights.

Table 2-3. ANOVA of seed weight (g) in eight populations of *Bonamia grandiflora* in the Florida scrub.

Source	df	SS	MS	F	P
Between populations	7	0.016	0.0020	21.284	< 0.05
Within populations	424	0.047	0.0001		
Total	431	0.063			

Seed length and width were also different between populations ($F = 6.15$, df = 4 and 150, $P < 0.05$ and $F = 12.54$, df = 4 and 150, $P < 0.05$, respectively; Tables 2-4 and 2-5, respectively). Duncan multiple-range comparisons showed a similar trend in both variables and similar to seed weight; Tower had the shorter and narrowest seeds and Sun Ray, the longest and widest ($\alpha = 0.05$; Fig. 2-6).

Overall, heavier seeds are also longer and wider seeds; however seed allometry was variable. The ratio of seed length to width was variable between populations ($F = 4.71$, df = 4 and 150, $P < 0.05$; Table 2-6). A Duncan multiple-range test showed that

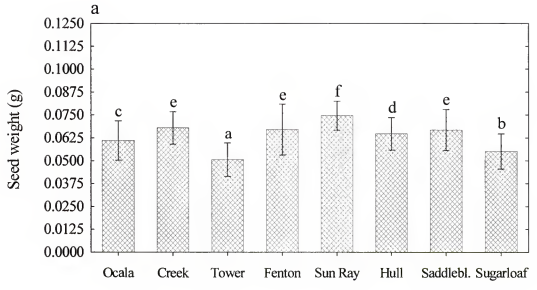


Figure 2-5. Seed weight (mean ± s.e.) in eight populations of *Bonamia grandiflora* in the Florida scrub. Populations with different letters are significantly different (Duncan multiple-range, $\alpha = 0.05$).

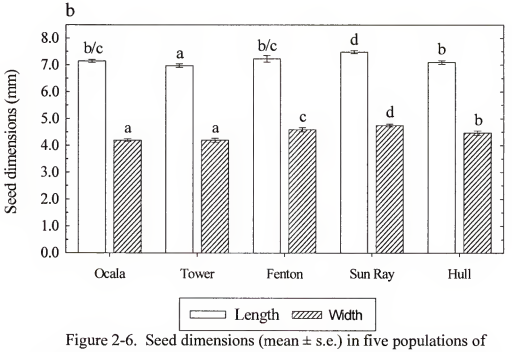


Figure 2-6. Seed dimensions (mean ± s.e.) in five populations of *Bonamia grandiflora* in the Florida scrub. Populations with different letters are significantly different (Duncan multiple-range, $\alpha = 0.05$).

Ocala had the highest ratio, 1.71 (Fig. 2-7). The overall mean ratio of seed length to width was 1.66 (± 0.18).

Table 2-4. ANOVA of seed length (mm) in five populations of *Bonamia grandiflora* in the Florida scrub.

Source	df	SS	MS	F	P
Between populations	4	4.39	1.10	6.15	< 0.05
Within populations	150	26.77	0.18		
Total	154	31.16			

Table 2-5. ANOVA of seed width (mm) in five populations of *Bonamia grandiflora* in the Florida scrub.

Source	df	SS	MS	F	P
Between populations	4	7.47	1.87	12.54	< 0.05
Within populations	150	22.33	0.15		
Total	154	29.80			

Table 2-6. ANOVA of the ratio of seed length (mm)/seed width (mm) in five populations of *Bonamia grandiflora* in the Florida scrub.

Source	df	SS	MS	F	P
Between populations	4	0.43	0.11	4.71	< 0.05
Within populations	150	3.44	0.02		
Total	154	3.87			

Germination percentages did not differ between populations (Kruskal-Wallis, $H = 6.44$, $\alpha = 0.05$; Fig. 2-8). However, there was a strong trend for the Ocala population

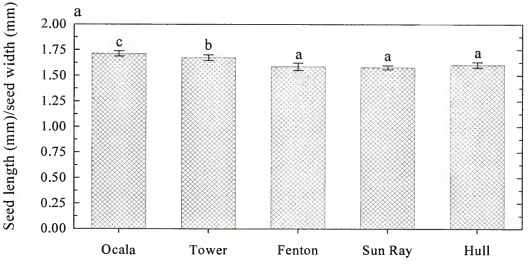


Figure 2-7. Ratio of seed length (mm) to width (mm) (mean ± s.e.) in five populations of *Bonamia grandiflora* in the Florida scrub. Populations with different letters are significantly different (Duncan multiple-range, $\alpha = 0.05$).

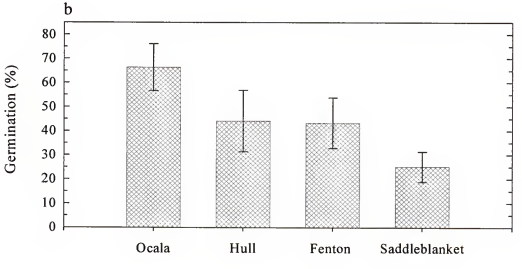


Figure 2-8. Percentage germination (mean ± s.e.) in four populations of *Bonamia grandiflora* in the Florida scrub.

to have the highest germination percentages and those of the Saddleblanket population, to have the lowest.

Overall, leaf pubescence (number of hairs/mm^2) was not different between the adaxial and abaxial leaf surfaces ($t = 0.14$, $df = 78$, $P = 0.44$). There was no difference between populations in leaf pubescence either in the adaxial or abaxial surfaces ($F = 0.41$, $df = 3$ and 36, $P = 0.75$; $F = 1.15$, $df = 3$ and 36, $P = 0.34$; respectively; Fig. 2-9).

Flower length and diameter did not differ between populations (both Kruskal-Wallis, $\alpha = 0.05$; Fig. 2-10). Depth of the corolla cleft did not differ between populations ($F = 1.61$, $df = 2$ and 27, $P = 0.22$; Fig. 2-10).

Discussion

The Tiger Creek Preserve populations had the plants with the fewest and shortest stems, the smallest plants, while one of the Saddleblanket Preserve populations had the plants with the most numerous and longest stems, the largest plants. The rest of the populations had intermediate values. While Saddleblanket had the largest plants, it did not have the largest reproductive output/stem unit. Reproductive output was not found to differ between populations; however Sun Ray tended to have higher values than the rest of the populations.

Both the largest and the smallest plants are located towards the southern end of the distribution, so there does not seem to be a geographic trend in plant size. The Tiger Creek populations appear subject to more shading than any of the others and this may explain the smaller size of the plants and their trend towards lower reproductive success.

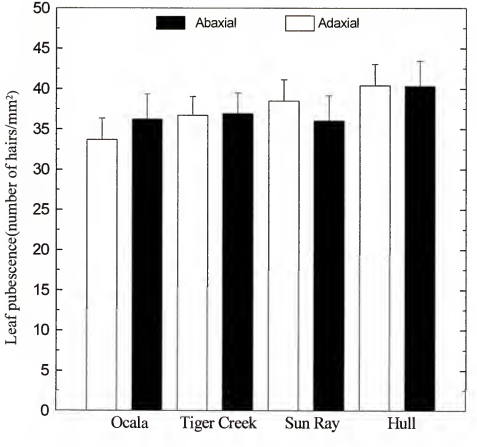


Figure 2-9. Pubescence, number of hairs/mm^2, (mean ± s.e.) in abaxial and adaxial leaf surfaces in four populations of *Bonamia grandiflora* in the Florida scrub. There was no difference between populations in the pubescence of the abaxial or adaxial leaf surfaces (ANOVA, $\alpha = 0.05$).

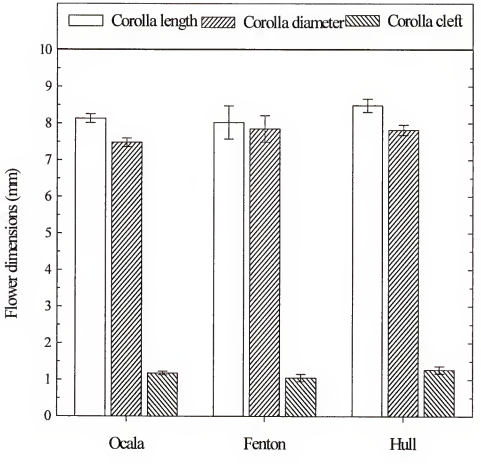


Figure 2-10. Flower dimensions, corolla length, diameter and depth of the cleft (mean ± s.e.) in three populations of *Bonamia grandiflora* in the Florida scrub. Corolla length and diameter were not different between populations (Kruskal-Wallis, $\alpha = 0.05$); corolla cleft was not different between populations (ANOVA, $\alpha = 0.05$).

As would be expected, plant size (expressed as total stem length) is positively associated with the number of fruits, flowers, and flower buds produced. It is, therefore, a good indicator of the reproductive potential of the plant and of the population as a whole. However, the regression model only explains approximately 50% of the total variation (although it is significant); this indicates that other factors influence the reproductive potential for each meter of stem; it is unknown if those factors are ecological or genetic or both. A weak but significant relationship between plant size and seed production was also found in *B. grandiflora* by Hartnett and Richardson (1989).

The reproductive output/m of stem did not differ between populations. This may indicate that if inbreeding depression is influencing this aspect of the reproductive potential of *B. grandiflora,* it is affecting all the populations studied evenly. However, the variances in these data were large and results must be interpreted with caution.

Sun Ray produced the heaviest and largest seeds, followed by Fenton whereas Tower had the lightest and smallest seeds. Ocala produced seeds of intermediate weight and intermediate to small size. However, Ocala had the highest ratio of seed length to width while Sun Ray and Fenton had low ratios. Seed weight and the ratio of length to width were not associated with total stem length, average stem length or percentage germination. There does not seem to be a geographic trend in seed weight, seed dimensions or in the ratio of length to width.

Ocala tended to have the highest percentage germination, Fenton and Saddleblanket, which had heavier seeds but lower ratios of seed length to width, tended to have lower percentage germination. This suggests that the ratio of seed length to

width rather than seed mass may be related to germination success. However, the seeds used in the germination experiments were not the same seeds used in seed allometry calculations, so this hypothesis was not tested directly.

There is no obvious geographic trend in any of the variables measured. Perhaps the observed variation depends on the characteristics of the microsite where each plant grows, the history of the site or individual plant. Percentage canopy cover, nutrient availability, precipitation, mean and extreme temperatures throughout the year, the age of the plants or their underground energy reserves are likely have an impact on plant size and reproductive output.

There was no *a priori* reason to think that small variations in flower dimensions would confer selective advantage. These characters may vary freely without impacting fitness. However, no significant variation was found in them and perhaps this means that there is little genetic variation underlying these characters due either to low overall genetic heterogeneity in *B. grandiflora* or to strong stabilizing selection that prevents even small variation.

Summary

Leaf pubescence, reproductive output/m of stem, percentage germination, and flower dimensions did not differ between populations. Number of stems/plant, stem length, seed weight, and seed dimensions and their ratio differed between populations. There is no obvious geographic pattern associated with any of the variables. Variation in plant size may result from habitat differences or random events or historical factors. Total reproductive output is associated with plant size but other (perhaps genetic) factors

have as much influence as size. The ratio of seed length/width may be related to the percentage germination and this relation should be investigated further.

CHAPTER 3
REPRODUCTIVE BIOLOGY OF *BONAMIA GRANDIFLORA*

Introduction

This study investigated the breeding system of *Bonamia grandiflora* (Convolvulaceae) by means of controlled pollinations of individual plants growing in three natural populations. The germinability of seeds resulting from the pollination experiment was tested to detect inbreeding depression. Other aspects of *B. grandiflora* related to its reproductive success were also investigated; natural pollinators were observed and seed herbivory was studied as well.

B. grandiflora is a perennial vine that produces large, bell-shape flowers in the summer and seeds abundantly. The flowers lack scent and are light purplish-blue and they normally possess five corolla lobes, five stamens and two stigmatic lobes. The throat of the corolla, stamens, pollen, and carpels are white; and the base of the corolla is greenish white. The anthers are introrse and release the pollen by longitudinal slits (Myint and Ward, 1968). The gynoecium has two carpels, fused at the ovary but with partially or almost totally free stylar branches (Myint and Ward, 1968). The ovary has two chambers, each one holding two ovules (Myint and Ward, 1968) although I have seen fruits with five and even six seeds. *B. grandiflora* has large flowers and accessible stigmas and anthers, this makes this species a good subject for mating system studies.

In a study of self-incompatibility in several species in the family Convolvulaceae, Martin (1970) found that most self-compatible species were annuals that flowered profusely over a short period. These species produce abundant seeds even when pollinators were excluded by the use of bags; in most of the species studied anthers dehisced before the flowers open (Martin, 1970). On the other hand, all the self-incompatible species studied were perennial; some of them were woody and others had fleshy storage roots. These self-incompatible species did not bloom abundantly and their seed set was poor, even in large populations. The stamens had variable length and the anthers dehisced extrorsely (Martin, 1970). A later study of *Ipomea pes-caprae* confirmed Martin's findings (Devall and Thien, 1989).

B. grandiflora is a perennial species and it has fleshy storage roots; however, it flowers and seeds profusely. It does not appear to fit Martin's classification of species in Convolvulaceae because it has characteristics of self-compatible as well as self-incompatible species and no predictions can be made about it based on his study. Given that *B. grandiflora* flowers are large, brightly colored, and open shortly after sunrise, and considering their morphology, cross-pollination is probably the most important means of producing seeds. However, because the flowers have introrse anthers and I have observed that, on occasion, a stamen may reach above a stigmatic lobe, self-pollination may be possible even without the aid of pollinators. Additionally, the lack of allozyme and DNA variation with RAPD markers (see Chapter 4) suggests that *B. grandiflora* may be normally selfing. Simultaneously, this prevented elucidation of the breeding system by molecular techniques. A mixed breeding system now appears to be the best hypothesis for *B. grandiflora*.

The Florida scrub, the habitat of *B. grandiflora*, is an ancient ecosystem. It has expanded and contracted over geological time and some scrub patches have been isolated for long periods. Although habitat fragmentation in the scrub has been accentuated by human activities, *B. grandiflora* populations have probably been partially isolated for thousands of years.

According to genetic models most of inbreeding depression is due to the accumulation of recessive lethal and semilethal mutations. These alleles could be eliminated by continued selfing at a low rate, sporadic pollinator failure or extreme population bottlenecks occurring more often than once every hundred generations (Lande and Schemske, 1985; Schemske and Lande, 1985).

Given the scrub geologic history, it is possible that many detrimental recessive alleles have already been purged out of the populations. It is hypothesized that little inbreeding depression will be found in selfed fruits and seeds of *Bonamia grandiflora*.

B. grandiflora seeds are ovoid and relatively large (approximately 0.06 g) and often they can be seen on the ground around mature plants, even in late spring of the following year. These seeds lack any obvious means of dispersal such as wings, pappus, or spurs; they are not ballistic and they appear to lack rewards for vectors such as elaiosomes or a fleshy tissue. *B.* grandiflora seeds do not appear to be very palatable to animals since they can be found in abundance. *B. grandiflora* seeds have relatively homogeneous seed coat color when they are extracted from capsules but they present a diversity of color when they are seen on the ground. This may indicate that the seeds remain on the ground for long times and become bleached over time. It was hypothesized that seed herbivory was low; this hypothesis was tested.

To assess pollinator availability, blooming *B. grandiflora* plants and their pollinators were observed. Observations were made in several populations and in different months and years.

Methods

Three populations in The Ocala National Forest, located in north central Florida were used in this experiment. Population 1 was beside forest route 88, between routes 88-B and 86; it is 5 km apart from population 2 and 12 km from population 3. Population 2 is at the intersection of routes 88 and 76 and approximately 5.5 km apart from population 3. Population 3 is along forest route 97 north of state highway 40.

Flower Morphology, Fruit and Seed Dimensions

Flower dimensions were measured in forty-five randomly chosen blossoms, one flower for each plant, in the three Ocala National Forest populations with a Spi vernier caliper. The variables measured were corolla length, corolla diameter, number of stamens and their length, and number of stigmatic lobes and the length of the carpels. In other species with tubular flowers, correlations were found among morphological measurements (Campbell, 1989; Campbell *et al.*, 1991) so the covariance of variables measured in *B. grandiflora* flowers was assessed.

To assess the presence of nectar guides *B. grandiflora* flowers were photographed with the aid of an Ilford Instrument Filter 828. This filter transmits U. V. radiation between approximately 300 and 400 nm and infrared between approximately 700 to 800 nm.

Fifty-nine randomly chosen capsules, one from each plant, from three populations were measured to obtain mean length and diameter. Length and diameter were also assessed in 54 seeds and weight was measured in 50. All the seeds were randomly chosen from five populations.

Pollination Biology

Flowers in the Ocala Seed Orchard, where the Forest Service personnel is growing *B. grandiflora* plants, and in wild populations 1, 2, and 3 were observed to assess pollinator activities. The observations were done in two seasons, the summer of 1992 and the summer of 1996, from 7 in the morning until 13:00 h. Only flowers that could be clearly seen at all times were included as focal flowers; i.e., the sample did not include the entire local plant population. Visits were noted only when pollination could have been performed by the insect; that is, the insect had to touch the anthers and stigmas while visiting the flowers. The number of visits of all pollinators per flower per hour was calculated for each hour of observation.

Seed Dispersal

To test the frequency of seed dispersal by animals, six groups of ten seeds were placed in each of the three Ocala wild populations. Because summer rains are heavy in the scrub and are capable of transporting seeds, screen boxes were used. These traps were open at the top to allow animals access to the seeds. The traps were buried in the sandy soil to provide a more natural environment and their sides protruded from the

ground two to three centimeters, to prevent the seeds from washing away during rainstorms. The traps were set in place in May 1996 and recovered in December 1996.

Hand-Pollination Study

Approximately 950 flowers were included in this study. Six pollination treatments were assigned an equal number of flowers, although the final sample sizes differed because of damage caused by vehicles trampling some plants and herbivore deer trampling. Natural pollinators were excluded by the use of brown paper bags. These were secured around the flower buds the day before the flowers opened. Early the following morning, these bags were removed and the flowers were either emasculated or pollinated or both with the aid of forceps. The stamens were seized by the filaments without disturbing the anthers. Stamens to be used for cross-pollinations were placed in new vials and refrigerated to avoid loss of pollen viability. After pollinations were performed, the bags were replaced or not, according to the treatment. The treatments were randomly assigned to the available flowers. The paper bags increased the temperature to which the flowers were exposed by 5°C in full sun; the increase in temperature was reduced or zero in cloudy or shady conditions. In preliminary trials this increase in temperature did not appear to influence fruiting success.

Four treatments involved emasculation of the flowers. The treatments were the following: 1) emasculation/controlled cross-pollination, 2) emasculation/controlled self-pollination, 3) emasculation/bagging to prevent pollination and 4) emasculation/open-pollination. The fifth treatment –bagging to prevent pollination- involved no further

manipulation. A control group was included; these flowers were not manipulated in any way. I counted the number of fruits produced and aborted, number of seeds/capsule, capsule weight, and seed weight in all treatments.

Breeding system

The mean numbers of seeds produced in fruits were used to calculate the self-incompatibility (SII) and the autogamy index (AI), according to the following formulae (Ramírez and Brito, 1990) :

$$SII = \frac{\text{number of seeds produced by self-pollinated flowers}}{\text{number of seeds produced cross-pollinated flowers}}$$

Self-compatible species have SII values between 0.30 and 1.00.

$$AI = \frac{\text{number of seeds produced by flowers bagged, but not emasculated}}{\text{number of seeds produced by self-pollinated flowers}}$$

Autogamic and partially autogamic species have AI between 0.30 and 1.00.

The selfing rate (S) was calculated from two measurements, the number of seeds per fruit and the percentage of germination for each treatment. The average number of seeds and average germination rates were used as estimates of fitness (w) for controls, emasculated/cross-pollinated, and emasculated/self-pollinated flowers (Charlesworth, 1988).

$$S = (w_{control} - w_{emasc./cross\text{-}pollinated})/(w_{emasc./self\text{-}pol.} - w_{emasc./cross\text{-}pol.})$$

Germination of the seeds produced by the pollination treatments was done in cell pack trays. One seed was planted in each cell in a mixture of native scrub soil and commercial sand (1:1). The location of seeds in the trays was randomized.

Statistical analysis

Appropriate programs of the SAS statistical package were used to analyze the data sets. Residuals were plotted against predicted values and treatments to assess their normality, homoscedasticity, and possible trends. Whenever necessary, data were transformed.

For most of the analyses mixed linear models were used, with pollination treatments as a fixed effect and populations and plants as random effects; capsules and seeds were replicates. Until the recent introduction of the procedures for mixed linear models, another one, GLM (General Linear Models) was used to analyze these fixed, mixed, and random model data sets. PROC GLM is designed for fixed effects although it contains a RANDOM option that produces an additional ANOVA table with the appropriate error terms. In mixed model procedures, however, the random effect is incorporated into all standard error calculations and tests (Litell *et al.*, 1996) so these were preferred to GLM.

In mixed linear models alternative procedures can be used, MIXED and VARCOMP. The former is based on the likelihood function (Litell *et al.*, 1996) and it provides parameter estimates, a test for the fixed effects, least square means and their comparisons, and linear combinations of means. The SATTERTH option was used to obtain a Satterthwaite approximation for the degrees of freedom associated with all estimates and tests in PROC MIXED. The VARCOMP procedure uses the method of moments and provides estimates of variance components, sums of squares, and the

expected mean squares. Note, however, that the F tests must be performed manually by choosing the correct ratios of mean squares according to the model used.

Fruiting success

An index was created to compare fruiting success between pollination treatments and populations. The fruiting success index was defined as follows:

$$\text{Fruiting success index} = \frac{\text{number of fruits per treatment} + 1}{\text{number of aborted fruits per treatment} + 1}$$

The traditional methods of analysis include χ^2 and G-tests on the number of fruits produced and aborted but these tests do not make efficient use of replicates, nor do they provide follow up analysis. For example, with a χ^2 or a G-test, only overall homogeneity of frequencies could be assessed, but not which treatments or populations differ from others. To achieve normality the indices were doubly transformed using the normal logarithm of their square root; both transformations have similar effects on the distribution of the data but only by using both of them was normalization achieved. A randomized complete block design was used with pollination treatments as a fixed effect and populations as blocks (random effect). Individual plants were used as replicates. PROC GLM was used to obtain estimates of population means and to perform Duncan's Multiple Range Test on their means since these options are not available with PROC MIXED or PROC VARCOMP.

Regression analysis was performed on mean fruiting success of each plant against the number of stems of the same plant. The number of stems in each plant is a good

indicator of plant size and it is a more expedient measure than quantifying the length of each stem.

Number of seeds in fruits, fruit weight, seed weight, and germination success

The number of seeds contained in each fruit, the weight of the fruits (capsules), and seed weight were compared between pollination treatments, populations, and plants. Mixed linear models were used with pollination treatments as a fixed effect, populations as blocks (random effect) and plants nested within populations also as random effects. Capsules were used as replicates for the number of seeds/fruit and fruit weight analyses, with seeds being the replicates in the seed weight analysis. To estimate and compare pollination treatment means PROC MIXED was used. To estimate population means the data were reanalyzed with PROC GLM and the RANDOM option, and Duncan's Multiple Range Test applied. PROC MIXED could not be used because means and differences can only be calculated for the fixed effect. To normalize their distribution seed weight data were transformed by raising them to the power 2.1; this power was estimated by SAS as the optimum.

To assess whether plant size is related to its reproductive success, regression analysis was performed on the mean number of seeds per fruit, mean fruit weight, and mean seed weight of each plant against the number of stems of the same plant.

Germination success data were not normal and no adequate transformation was found; a Friedman's non-parametric two-way test was performed first by ranking the data by block (PROC RANK) and then using PROC GLM for fixed effects; type III

sums of squares were selected. To detect differences between populations the data were re-analyzed disregarding pollination treatments. A non-parametric one-way ANOVA was used.

Results

Flower Morphology, Capsule and Seed Dimensions

The blooms appear to absorb ultraviolet light in the throat and base of the corolla; these are the white areas of the flower as seen in daylight and where the nectaries are located (Fig. 3-1). The anthers are highly reflective. This contrast may attract U.V. sensitive pollinators such as bees.

Average corolla diameter was 76.55 mm (± 6.43), the average corolla length was 80.07 mm (± 4.30), average stamen length was 48.68 mm (± 3.07), and average carpel length was 54.32 mm (± 3.93). All the stamens are shorter than the styles in approximately 80% of the flowers, in the remaining 20% one of the styles is shorter than one of the stamens. Typically, the flowers have two stylar branches, but approximately 9% of the flowers have a third branch.

Average stem length and average carpel length were weakly, but significantly associated with corolla length ($r^2 = 0.44$, $P < 0.05$; $r^2 = 0.20$, $P < 0.05$, respectively). Corolla diameter was not associated with any other flower dimension.

A few flowers were observed to have six corolla lobes and one flower in the sample had seven lobes. The latter had a longer corolla than the average, 91mm, but the diameter was normal, 73.2 mm. These six-lobed flowers were observed in two of the populations in at least two plants; those flowers were not part of the sample. These

Figure 3-1. *Bonamia grandiflora* flowers. The two photographs at the top were taken without special filters. The two photographs at the bottom were taken with an Ilford Instrument Filter 828. This filter transmits U. V. radiation between 300 and 400 nm and infrared between 700 and 800 nm. The throats of the corollas show increased absorption of U.V. wavelengths and the androecium and gynoecium appear to reflect those wavelengths.

plants produced several six-lobed flowers but they also produced normal flowers. Of the forty-five flowers measured, four had a third stigmatic lobe; this was shorter than the other two. There were no differences in corolla length, corolla diameter, stamen length, and stigmatic lobes length between the three populations ($p < 0.05$ in all tests).

Pollination Biology

The mean number of visits by pollinators per flower per hour was highly variable ranging from 0 to 9.5. All the observations were made between 7:00 and 13:00 h. *B. grandiflora* flowers start opening before dawn but many of them are not fully opened until well past 7:00. Corollas show considerable wilting after 13:00 h. and pollinators seem to decrease their activity after noon. Pollinator activities peaked at somewhat different times on different days but overall most visits occurred between 9:30 and 11:30 h. The rate of visitation differed between June, July, and August ($F = 8.99$, $df = 2$ and 34, $P > 0.001$).

In 1992, seven hours of pollinator observation were done at the Seed Orchard and fourteen were done in population 2 and 3. The mean visitation rate was 0.68 ($\pm$ 0.59) visits per flower per hour. The observations in the wild populations were done in July and August. Towards the end of the summer the availability of pollinators seemed to decrease.

In 1992, there was an abundance of colletid bees and sulfur butterflies both in the Ocala Seed Orchard and in populations 2 and 3 in the Ocala National Forest but those

pollinators were practically absent in 1996 from populations 1 and 3. However, colletid bees were seen pollinating palmetto flowers (*Serenoa repens*), in 1996.

In 1996, twenty-eight hours of observation produced a mean visitation rate of 1.65 visits/flower x h (± 2.43). At the beginning of the blooming season, during mid-June visitation rates were 0 or very low. The very fragrant palmetto flowers seemed to attract large and small bees, bumble bees, wasps, beetles, and ants while *B. grandiflora* flowers in the vicinity went unvisited. Only swallowtail butterflies visited *B. grandiflora* but not palmettos while the latter were in bloom. However, butterflies and skippers were not abundant at that time and many of them flew over *B. grandiflora* plants without visiting their flowers; it is suspected that flowers that are more attractive are available at the beginning of the summer.

Later in the season, in mid-July, the overwhelming majority of visits was by swallowtail butterflies: palamedes and pipevine; on average they were responsible for over 78% of the visits (± 20). These butterflies tended to stay in the same area and to visit the flowers repeatedly. The pipevine swallowtail chased other insects including the palamedes swallowtail; one individual pipevine swallowtail (as identified by damage in the wings) was observed during two consecutive days in the same *B. grandiflora* population. Another common visitor of *B. grandiflora* blooms, a flower fly, tended to visit flowers in close proximity to each other and to perch on stigmas and stamens for long periods. Other pollinators such as bees, bumble bees, wasps and beetles visited flowers briefly, and visited fewer flowers before leaving the *B. grandiflora* populations; these insects appear more likely to carry pollen over longer distances.

The list of pollinators is by no means exhaustive since a relatively small number of hours were spent on these observations but the following insects were observed on *B. grandiflora* flowers: pipevine swallowtail, *Battus philenor*; palamedes swallowtail, *Papilio palamedes*; cloudless sulfur, *Phoebis sennae*; the first two butterflies are in the Papilionidae family and the last is a Pieridae. Folded-wing skippers, Hesperiidae, subfamily Herperiinae, the yehl skipper, *Poanes yehl*; clouded skipper, *Lerema accous*; the fiery skipper, *Hylephila phyleus*, and another unidentified skipper also visited *B. grandiflora*. Flower flies in the Syrphidae family were frequent visitors in 1992 and 1996, and small bees (Colletidae) were common only in 1992. Less common visitors were bumble bees, *Bombus* sp. (Bombicidae), large Vespidae wasps, bee flies (Bombyliidae) and a few unidentified small beetles.

Seed Dispersal

After seven months in the field 65% of the seeds were recovered. One trap was destroyed, probably trampled by deer, and it was not included in the analysis. Empty seed coats were found in two of the traps. However, other seed coats could have been present in other traps since the coats could easily pass through the sieve that was used to recover the seeds. There were no indications of what animals could be responsible for the disappearance of seeds. Perhaps beetles consumed the cotyledons and embryos leaving the hard and nutritionally poor seed coat.

Hand-Pollination Study

Breeding system

All the pollination treatments produced fruits and seeds. *B. grandiflora* is highly self-compatible as indicated by a SII of 0.875. It is also autogamic, that is, it can self-pollinate, as shown by an AI of 0.734. It is agamospermic or apomictic, because it can produce seeds without pollination.

Two estimates of the selfing rate were obtained. The selfing rate was 0.68 when estimated from the number of seeds/fruit and 0.49 when the percentage of germination was used.

Table 3-1. Number of seeds recovered after seven months for each of the six traps in three *Bonamia grandiflora* populations in the Ocala National Forest. Each trap originally had ten seeds.

Population	Trap #	Seeds recovered	Population	Trap #	Seeds recovered
OC. 1	1	8	OC. 2 (cont.)	4	6
	2	8		5	6
	3	9		6	3 *
	4	3	OC. 3	1	9
	5	6		2	8 *
	6	0, trap destroyed		3	7
OC. 2	1	7		4	5
	2	6		5	6
	3	5		6	9

* Empty seed coats were also found in these traps.

Fruiting success

Pollination treatments differed in fruiting success ($F = 21.85$, $df = 5$ and 221, $P<0.001$, Table 3-2). Pairwise comparisons of the least significant means showed that the bagged and emasculated/bagged treatments were different from all the others (all $P<0.001$ and $df = 221$; Fig. 3-2a).

Populations differed in fruiting success ($F = 6.99$, $df = 2$ and 221, $P<0.001$; Table 3-2). Population 2 had the highest fruiting success index, the other two were not different from each other (Duncan's Multiple Range test, $\alpha = 0.05$; Fig. 3-2b). The variance component for populations was 0.0024.

The size of the maternal plant did not seem to affect the fruiting success. There was no association between the fruiting success and the number of stems of each individual plant ($r^2 = 0.024$,).

Table 3-2. ANOVA of fruiting success index in six pollination treatments (control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated) and three populations of *Bonamia grandiflora*. The sums of squares and mean squares were calculated with PROC VARCOMP in SAS.

*Fruiting success index = (#of fruits per plant +1)/(#of aborted fruits per plant + 1)

Source	df	Type I SS	Type I MS	F	P
Pollination	5	2.927	0.585	21.85	P<0.001
Populations	2	0.374	0.187	6.99	P<0.001
Error	221	5.921	0.027		
Corrected total	228	9.223			

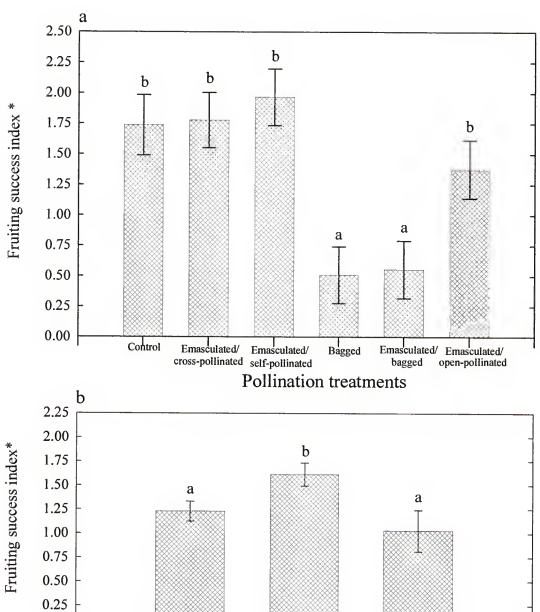


Figure 3-2. Fruiting success in a hand-pollination study of *Bonamia grandiflora* in the Ocala National Forest. Different letters indicate a significant differences between treatments or populations.

a. Fruiting success (means ± s.e.) for six pollination treatments.

b. Fruiting success (means ± s.e.) for three populations

* fruiting success = (# of fruits per plant + 1)/ (# of aborted fruits per plant + 1)

Number of seeds in fruits

Pollination treatments produced different numbers of seeds per capsule ($F = 5.17$, $df = 5$ and 239, $P<0.001$; Table 3-3). The emasculated/cross pollinated treatment differed from the emasculated/self-pollinated ($t = 2.39$, $df = 274$, $P<0.05$), the bagged ($t = 3.52$, $df = 291$, $P<0.001$), the bagged/emasculated ($t = 3.26$, $df = 285$, $P<0.005$), and the emasculated/open-pollinated. ($t = 3.43$, $df = 282$, $P = 0.001$), (Fig. 3-3a).

Whereas there was no difference between populations (Fig. 3-3b), there was a difference between plants within populations in the number of seeds produced per capsule ($F = 2.35$, $df = 53$ and 239, $P<0.001$; Table 3-3).

The variance component for populations was –0.0769 and the one for plants was 0.2849. There was no association between the average number of seeds per fruit and the size of the maternal plant expressed as number of stems ($r^2 = 0.00009$).

Table 3-3. ANOVA of the number of seeds per fruit in six pollination treatments (control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated) and three populations of *Bonamia grandiflora*. The sums of squares and mean squares were calculated with PROC VARCOMP in SAS.

Source	df	Type I SS	Type I MS	F	P
Pollination	5	22.388	4.478	5.17	P<0.001
Populations	2	1.091	0.546	0.27	N/S
Plants	53	107.995	2.038	2.35	P<0.001
Error	239	207.123	0.867		
Corrected total	299	338.597			

Fruit weight

Capsule weight was different between pollination treatments ($F = 2.73$, $df = 5$ and 246, $P<0.01$; Table 3-4). The control differed from the bagged treatment ($t = 2.63$, $df =$

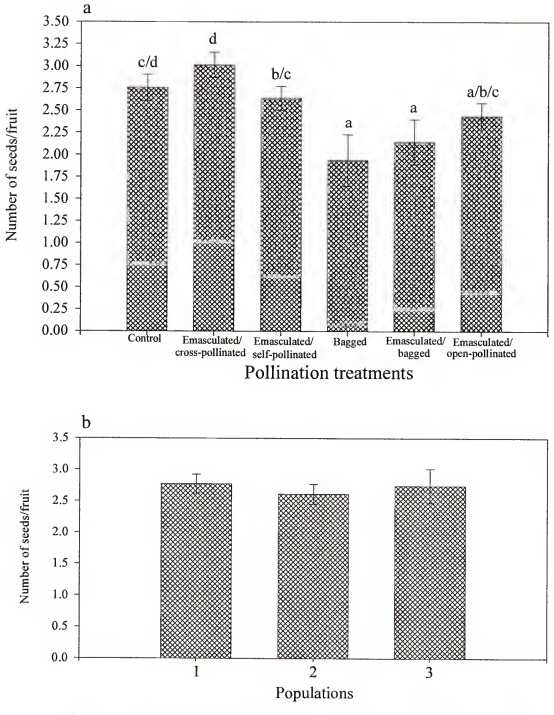


Figure 3-3. Number of seeds in each fruit in a hand-pollination study of *Bonamia grandiflora* in the Ocala National Forest.

a. Seeds/fruit (means ± s.e.) for six pollination treatments.

b. Seeds/fruit (means ± s.e.) for three populations.

274, P<0.01; Fig. 3-4a). The emasculated/cross-pollinated treatment differed from the bagged (t = 2.60, df = 283, P<0.01; Fig. 3-4a). However, the emasculated/cross-pollinated and emasculated/self-pollinated treatments were not different.

There were differences between populations (F = 3.96, df = 2 and 51, P<0.05; Table 3-4) and among individuals (F = 2.39, df = 51 and 246, P<0.001; Table 3-4). Population 1 had the highest fruit weight, the other two were not different from each other (Fig.3-4b).

The variance component for populations was 0.000016, for individual plants it was 0.0016. There was no association between the plant size expressed as number of stems and average fruit weight ($r^2 = 0.014$).

Table 3-4. ANOVA of fruit weight in six pollination treatments (control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated) and three populations of *Bonamia grandiflora*. The sums of squares and mean squares were calculated with PROC VARCOMP in SAS.

Source	df	Type I SS	Type I MS	F	P
Pollination	5	0.067	0.013	2.73	P<0.01
Populations	2	0.093	0.046	3.96	P<0.05
Plants	51	0.598	0.012	2.39	P<0.001
Error	246	1.206	0.005		
Corrected total	304	1.965			

Seed weight

Seed weights differed between pollination treatments (F = 10.64, df = 5 and 636, P<0.001, Table 3-5). Seeds from the bagged and emasculated/bagged treatment had the highest average weight (Fig. 3-5a). The control differed from all other treatments except for the emasculated/open-pollinated (Fig. 3-5a). The emasculated/cross-pollinated

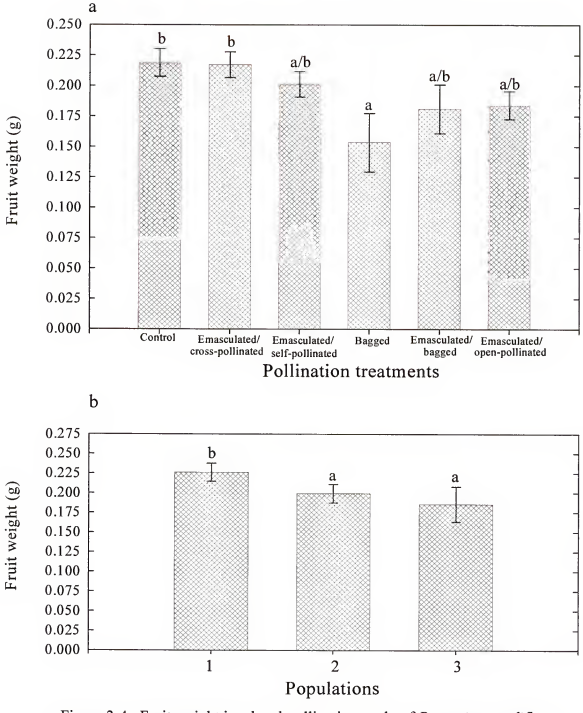


Figure 3-4. Fruit weight in a hand-pollination study of *Bonamia grandiflora* in the Ocala National Forest.

a. Fruit weight (means ± s.e.) for six pollination treatments.

b. Fruit weight (means ± s.e.) for three populations.

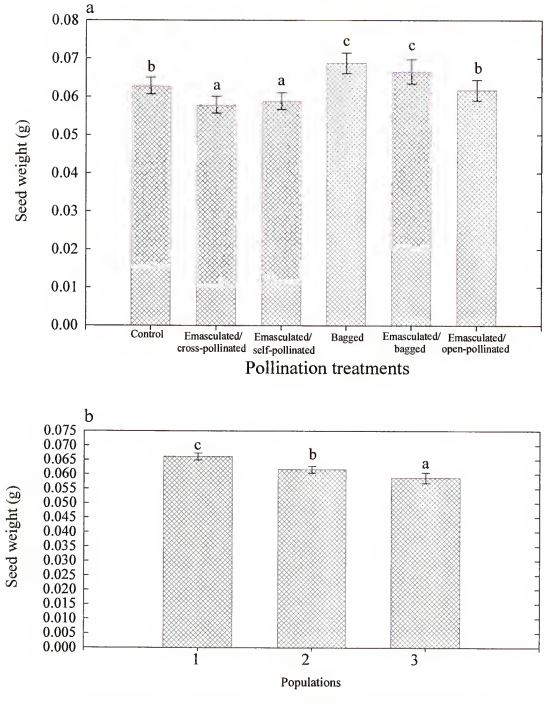


Figure 3-5. Seed weight in a hand-pollination study of *Bonamia grandiflora* in the Ocala National Forest..
a. Seed weight (mean ± s.e.) for six pollination treatments.
b. Seed weight (mean ± s.e.) for three populations.

treatments differed from all others except for the emasculated/self-pollinated (Fig. 3-5a).

Seed weights differed between populations ($F = 5.71$, $df = 2$ and 48, $P<0.01$; Table 3-5), among individual plants ($F = 1.86$, $df = 48$ and 65, $P<0.01$; Table 3-5), and capsules ($F = 3.91$, $df = 65$ and 636, $P<0.001$; Table 3-5). Population 1 had the highest seed weights, population 3 the lowest and population 2 was intermediate (Fig. 3-5b).

Table 3-11. ANOVA of the transformed seed weight* in six pollination treatments (control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated) and three populations of *Bonamia grandiflora*. The sums of squares and mean squares were calculated with PROC VARCOMP in SAS.

Source	df	Type I SS	Type I MS	F	P
Pollination	5	0.00003725	0.00000745	10.64	P<0.001
Populations	2	0.00005836	0.00002918	5.71	P<0.01
Plants	48	0.00024522	0.00000511	1.86	P<0.01
Capsules	65	0.00017797	0.00000274	3.91	P<0.001
Error	636	0.00044776	0.00000070		
Corrected total	756	0.00096656			

*seed weight$^{2.1}$

The variance component for populations was 0.0000004, for individual plants and 0.0000003 for capsules. There was no association between plant size expressed as number of stems per individual and average seed weight for the same individual ($r^2 = 0.033$, $P > 0.05$). There was a strong negative association between the average transformed seed weight for a treatment and the average number of seeds/capsule for the same treatment ($r^2 = 0.85$, $P < 0.05$).

Germination trials

Seedling emergence took 10 to 15 days. The percentages of seed germination are low; they range from 17.85% for the bagged only treatment to 52.17% for the emasculated/cross-pollinated (Fig. 3-6a). There was no statistical difference in the ranks of percentage of seed germination between pollination treatments ($F = 1.17$, $P = 0.33$; Fig. 3-6a). However, germination tended to be much lower in the bagged only treatment and higher in the emasculated/cross-pollinated (Fig. 3-6a).

There was a difference between the ranks of the populations and between plants, but not among capsules. However, because the data were ranked by population, a significant difference between them only reflects a difference in sample size; the more samples, the higher the rank and therefore the higher the mean, and not a true difference in germination percentage. A subsequent non-parametric ANOVA disregarding pollinations treatments shows no differences between population means ($F = 1.953$, $df = 2$, $P > 0.05$, Fig. 3-6b) despite the trend of a higher mean in population 2 (Fig. 3-6b).

Many plants had germination percentages of zero while others reached 100%. This wide variation was encountered in the three populations. There was no association between plant size expressed as number of stems and the germination percentage. However, there was a very strong association between the average percentage germination for a treatment and the average number of seeds in the capsules in the same treatment ($r^2 = 0.91$, $P < 0.05$). Since number of seeds/capsule and seed weight had a negative association, there was also a negative association between the average

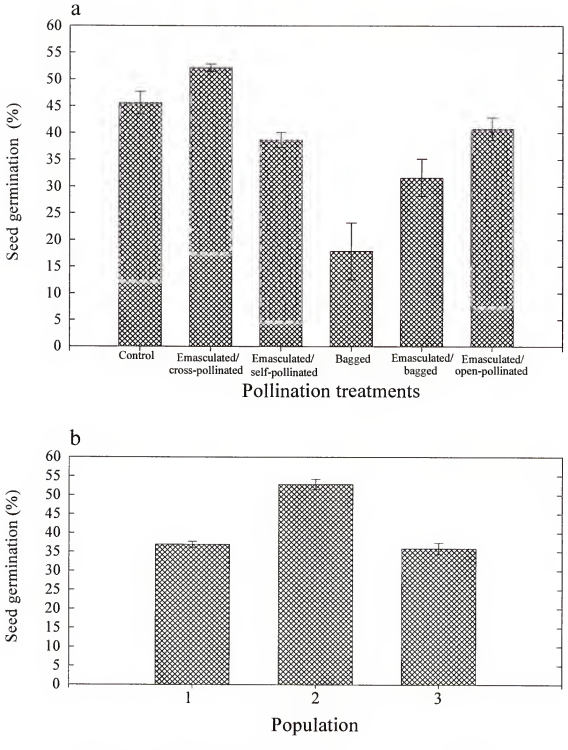


Figure 3-6. Percentage germination in a hand-pollination study in *Bonamia grandiflora* in the Ocala National Forest.

a. Percentage germination (means ± s.e.) for six pollination treatments.

b. Percentage germination (means ± s.e.) for three populations.

percentage germination for a treatment and the average transformed seed weight for the same treatment ($r^2 = 0.80$, $P < 0.05$).

Discussion

Fruit and seed formation were possible in all of the pollination treatments. Self-fertilization was possible even in the absence of pollinators (bagged treatment) and so was apomixis (fruit formation without fertilization -emasculated/bagged treatment-).

In fruiting success, all treatments that involved pollination by hand or a pollinator, the emasculated/cross-pollinated (1.777 ± 0.227), emasculated/self-pollinated (1.965 ± 0.231), control (1.737 ± 0.247) and emasculated/open pollinated (1.376 ± 0.0.233) were different from the treatments that involved no pollinating agent, the bagged (0.508 ± 0.0.232) and emasculated/bagged (0.554 ± 0.236) (Fig.3-2a). This suggests that pollination vectors are important to ensure seed set. *B. grandiflora* does not require a specialized pollinator so it is not susceptible to the loss of any particular insect species. However, it appears that pollinators may be scarce for this species when flowers that are more attractive are available.

The remaining analyses, number of seeds/fruit, fruit weight, seed weight, and percentage germination, of course, consider only the fruits and seeds that successfully developed so these results must be interpreted within this context. Because there is no difference in fruit weight between the bagged, emasculated/bagged, emasculated/self-pollinated and emasculated/open-pollinated treatments, it is clear that the few fruits which successfully developed in the bagged and emasculated/bagged reached a normal

weight. Therefore, the principal effect of these pollination treatments is the reduction in the number of fruits, while other variables are not necessarily affected.

Fruit weight was relatively homogeneous between treatments (Fig.3-4a). The only significant differences were found between the bagged (0.153g ± 0.011) and the cross-pollinated/emasculated (0.217g ± 0.010) and control (0.219g ± 0.011) (Fig.3-4a).

The number of seeds per fruit and seed weight had an inverse relationship; that is, the treatments that had the highest number of seeds, the emasculated/cross-pollinated, control, and emasculated/self-pollinated treatments had the lightest seeds (Fig. 3-3a and 3-5a). The bagged and emasculated/bagged treatments produced fewer but heavier seeds (Fig. 3-3a and 3-5a). Apparently, similar biomass is invested in each one of the fruits as shown by the fruit weight analysis, but the allocation differs; fewer and heavier seeds are produced in the treatments in which pollination was not performed. Similar trends were found in *Zephyranthes atamasco*, the Atamasco lily from Georgia and Florida; self- and cross-pollinations produced larger number of seeds in each fruit than open-pollinations but fruit weight was relatively constant (Broyles and Wyatt, 1991). A negative correlation between mean seed mass and number of seeds per fruit has been found in several other species (Harper *et al.*, 1970; Méndez, 1997; Obeso, 1993), but not in all (e.g., Lokker and Cavers, 1995; Obeso, 1993). In *B. grandiflora* this trade off between seed number and mass could be caused by genetic differences among the seeds in a fruit, mother/offspring conflict or sibling rivalry as it has been suggested in other species (Temme, 1986; Uma Shaanker *et al.*, 1988).

The number of seeds/capsule in the control treatment was 2.76 (± 0.15). This value is similar to those calculated for 1985 and 1986 for Ocala populations, approximately 2.7 and 2.5 seeds/capsule, respectively (Hartnett and Richardson, 1989).

Overall, the percentage of germination is low. While there is great variability between plants, this variation is not associated with plant size. There is a strong trend toward low percentage of seed germination in the bagged only treatment and high values in the emasculated/cross-pollinated (Fig. 3-6a).

Although the emasculated/cross-pollinated treatment had the lowest seed weights (Fig. 3-5a), this treatment had the highest germination (Fig. 3-6a). Even though differences in germination percentage were not significant, the regressions of percentage germination over the number of seeds/capsule and transformed seed weight were very strong and unlikely to have occurred by chance (probability of Type I error = 0.0029 and 0.0170, respectively)

Because heavier seeds have more energy reserves, it was thought that they would be more likely to germinate. However, these results are not unprecedented; similar results have been found in *Zephyranthes atamasco* (Broyles and Wyatt, 1991), two alpine *Saxifraga* species by Gugerli (Gugerli, 1997) and in another *Saxifraga* species in Denmark (Dahlgaard and Warncke, 1995). It has also been documented in *Abutilon theophrasti* (Zhang and Hamill, 1997).

It appears that in *B. grandiflora* the number of seeds per fruit is a more important predictor of germination success than seed weight because both may have the same underlying causes. It is possible that the heterosis produced by cross-pollinations overcomes the disadvantage of small seeds. If this is true, this heterosis would also

show in open-pollinated and control fruits; and indeed it does, but to a lesser degree. This could be explained by a difference in pollen loads achieved on stigmatic lobes in these treatments. The flowers that were hand-pollinated had the stigmas totally covered by pollen, while the flowers open to natural pollinators did not reach these high pollen loads. The difference between the control and open-pollinations is consistent with this explanation since the control flowers had intact anthers but the open-pollinated flowers were emasculated.

Scrub endemics present a variety of reproductive systems, *Eryngium cuneifolium* is protandrous and self-compatible but not agamospermous (Menges, *et al.* 1996). *Hypericum cumulicola* and *Warea carteri* are also self-compatible and not agamospermous although the flowers are not protandrous (Evans, *et al.*, 1996; Menges, *et al.* 1996). *Liatris ohlingerae* is self-incompatible, not autogamous and there is spatial and temporal separation of the sexual function in the flowers (Menges, *et al.* 1996). In *Nolina brittoniana,* most of the plants are either male or female at a particular time during the blooming season (Menges, *et al.* 1996). *B. grandiflora* appears to be more similar to *H. cumulicola* and *W. carteri* than to the other species studied, not only in the characteristics mentioned above but also in showing little inbreeding depression in the early stages of the life cycle.

The selfing rate (S), the ratio of the difference between a control and the emasculated/cross-pollinated and the difference between the emasculated/self-pollinated and emasculated/cross-pollinated, provides an estimate of inbreeding depression; the smaller the rate the greater the effect of inbreeding depression. The two estimates of selfing rate for *B. grandiflora* do not agree very closely (0.68 by the number of

seeds/fruit and 0.49 by the germination rate) but they both indicate that some inbreeding depression is likely in this species. This discrepancy could be due to the inbreeding depression having additional effects during germination as would be expected. That is, as the plant develops, more genes are expressed and each one of them has additional probabilities of showing inbreeding depression. On the other hand, variation in selfing rates has also been found in other species such as *Vaccinium myrtillus*, *V. vitis-idaea* (Jacquemart and Thompson, 1996), and in several species of gymnosperms and other angiosperms (Charlesworth and Charlesworth, 1987). As is the case in *B. grandiflora*, the selfing rates in these species were based on more than one variable, such as number of seeds/fruit and germination rate. It has been thought that most species are either primarily outcrossing ($S < 0.2$) or primarily selfing ($S > 0.8$) (Schemske and Lande, 1985). *B. grandiflora*, however, shows intermediate S values (0.49 and 0.68).

In angiosperms, inbreeding depression has been shown to be mainly expressed in the stages of growth and reproduction (Husband and Schemske, 1996). Although the effects of inbreeding depression are undetectable or mild in *B. grandiflora* in the developmental stages studied here, it is probable that in later stages inbreeding will have stronger effects. If the magnitude of inbreeding depression encountered in later stages of *B. grandiflora* is comparable to the figures shown in Husband and Schemske (1996), less than five times more depression than that found already should be expected.

Number of seeds and germination percentage have also been compared directly between the emasculated/self-pollinated and emasculated/cross-pollinated. Only the first comparison showed significant differences and therefore inbreeding depression. In the

germination study, the emasculated/self-pollinated treatment had non-significantly lower values than those in the emasculated/cross-pollinated.

It was observed that pollinators, especially butterflies, visiting *B. grandiflora* flowers tended to visit each and every flower, sometimes more than once. Because of this butterfly behavior, it was expected that facilitated self-pollination and geitonogamy (Schoen and Lloyd, 1992) would occur very frequently. Because some inbreeding depression seems to affect selfed fruits and seeds it was expected that facilitated self-pollination would diminish the fitness of those individuals as has been shown in other species. This hypothesis was not directly tested but some inferences can be made from the data available. In *Geranium caespitosum* it was shown that geitonogamously (within plant) pollinated plants had lower fruiting success, and lower average seed weight (Hessing, 1988). In the same study, it was also shown that geitonogamous pollinations occurred more often in larger plants, which had more flowers open at the same time. This observation does not seem to hold true in the case of *B. grandiflora.* Control flowers were left unmanipulated and free to be visited by natural pollinators; larger plants of *B. grandiflora,* as in *Geranium caespitosum,* have more flowers and pollinators tend to visit several flowers in each plant. It was expected that control flowers would produce the lowest values in the variables measured in the population with the largest plants. Population 3, which had the largest plants, showed a trend towards the lowest percentage of germination in the control flowers, which would support the hypothesis. However, the same trend was found in the cross-pollinated treatment, which would not have been expected since all the cross-pollinations were done between populations. Whatever factor decreases fitness in population 3, does so evenly across treatments.

There was no relationship between any of the variables measured and plant size expressed as the number of stems. This lack of association with plant size was also found in *Gentianella germanica* (Fischer and Matthies, 1997). It appears that the plants capable of flowering have enough energy reserves in their roots to support the development of the seeds.

Comparing the bagged only (obligated self-pollination) and emasculated/bagged (apomixis) treatments shows no significant differences. However, the emasculated/bagged treatment had a success index 10% higher, an average fruit weight 16% higher, and a percent germination 44% higher than bagged only. This suggests that emasculation may trigger seed development.

To gain some insight into the effects of natural pollinators, two of the treatments can be compared: the control (no manipulation) and the emasculated/open-pollinated flowers. Control flowers can be cross-pollinated or self-pollinated with the help of pollinators, they can self-pollinate without the intervention of pollinators or even produce seeds by apomixis. Emasculated/open-pollinated can only be cross-pollinated or form seeds apomictically. The occurrences of successful apomixis (emasculated/bagged treatment) and spontaneous self-pollination (bagged treatment) are very low (Fig. 3-2a) and their effect on the control or open-pollination treatments is probably small. The comparison of control and the emasculated/open-pollinated flowers provides an estimation of cross-pollination performed by pollinators. While none of the variables was significantly different between these two treatments, the fruiting success index was substantially lower in the emasculated/open-pollinated than in the control. The number of seeds/fruit was not different between open-pollination and control but

while the open-pollination was different from the cross-pollination, the control was not. This suggests that pollination with self pollen may occur frequently.

In natural pollinations vectors may or may not be present in sufficient numbers between dawn and one or two o'clock in the afternoon, and pollinators may or may not be able to carry sufficient amounts of pollen. An indication of pollinator abundance and effectiveness can be seen in the comparison between control and the emasculated/cross-pollinated treatments. In the emasculated/cross-pollinated treatment the stigmas were completely covered with pollen. In the present study, the results of the controls were not different from the emasculated/cross-pollinated treatment; this would indicate that there is no significant shortage of pollinators or pollen being deposited on the stigmas.

Population 1, the northernmost one in the study and almost the limit of the geographic range of the species, had the highest fruit and seed weight. This population was also the first to start blooming and the largest one. Population 2 had the highest fruiting success and tended to have the largest germination percentage. This second population was located at an intermediate latitude; it also had an intermediate number of individuals and started blooming later than population 1 but before population 3. Population 3 had the lowest values for all the variables measured; it was the southernmost population and also the smallest one, but it had the two largest individuals. Because the geographic location and population sizes are confounded, it is impossible to speculate which one, if any, has a causal relationship to the results of the experiment. Additionally, it is unknown if geographic location has an effect at this scale (all the populations were located in the Ocala National Forest) although the difference in phenology suggests that it may be possible.

Population size has been shown to be an important factor in the reproduction of some species such as *Gentianella germanica* in which small populations had smaller plants that produced fewer fruits, and fewer seeds per fruit than plants in larger populations (Fischer, 1996; Fischer and Matthies, 1997). This, apparently, is not the case in *B. grandiflora* but this hypothesis has not been tested directly.

B. grandiflora seeds have a hard seed coat. This impermeable seed coat is probably similar to those of other Convolvulaceae (Rolston, 1978). This type of seed coat consists of a palisade layer of macrosclereid cells impregnated with suberin, cutin, lignin and other water-repellent substances (Rolston, 1978). Other species with this physical dormancy germinate when exposed to particular temperature fluctuations. This has been observed both in trees such as *Heliocarpus donnell-smithii* (Vázquez-Yanes and Orozco Segovia, 1982) as well as in herbaceous species such as *Trifolium subterrraneum* (Taylor, 1981) among others. It appears that release of seed dormancy in these species is not due to microbial destruction of the seed coat or its scarification as it was once thought but that particular environmental conditions affect seed coat permeability (Baskin and Baskin, 1989).

A large number of seedlings were observed emerging in one of the Ocala populations at approximately the same time (358) so it seems that environmental conditions may be releasing the seeds from their dormancy. The following season 100 new seedlings were found in the same population. In contrast, the other Ocala populations produced a few seedlings during the first season and none in the second. It is unknown what particular conditions could have triggered such high germination. However, seedling recruitment is normally low, for example Hartnett and Richardson

(1989) reported 0.14 seedlings/100 m^2 in an unburned site and 15 seedlings/100 m^2 in a burned one. Unless the environmental conditions that release dormancy are microsite specific, when only a few seedlings germinate the trigger is more likely to be weakening of the seed coat by microorganisms or scarification. Perhaps all of these mechanisms occur in *B. grandiflora* populations producing poor recruitment during most seasons and mass germination on rare occasions. The year following the germination of 358 seeds only 27% of the seedlings survived (96). However, even if one fourth of those 96 year-old seedlings survived to reproduction, it would produce a substantial increase in that population. If environmental conditions were adverse following mass germination, it would cause losses to the seed bank but it would not deplete it, since only buried seeds germinate.

Fire seems to scarify the seeds and promote germination in *B. grandiflora.* This aspect was not tested in this research although, as mentioned before, Hartnett and Richardson (1989) found a large difference in seedling recruitment between burned and unburned areas.

There was a 5% seed loss/month in seed traps, and no animals were found in capsules. This contrasts to Hartnett and Richardson's study, in which they found that 56% of the plants studied in an Ocala population had capsule and seed damage caused by insect larvae. Instead, extensive fungal damage was observed in capsules and seeds during 1995. The fruits were attacked before the seed coats had hardened so mortality was practically 100% in the pollination study conducted that season. During 1996, there were no losses due to fungal infestation.

One way in which fire may help in seed dispersal is through increased surface runoff after rains when above ground matter and litter have diminished (Ahlgren and Ahlgren, 1960; Humphreys and Craig, 1981; Whelan, 1986). The heavy summer rainfall in the Florida scrub is certainly capable of transporting *B. grandiflora* seeds, and it was the reason why screen traps were constructed for the seed herbivory study. Additionally, rainfall washes organic matter into small depressions and can form "microterraces" around obstructions (Whelan, 1986). I have observed the latter in unburned areas as well. These sites could provide much needed nutrients to young plants. However, no seedlings were observed emerging from depressions or microterraces with litter; perhaps these areas are later covered by sand.

Summary

Bonamia grandiflora has a mixed mating system, and it can be apomictic. However, pollinators are essential to ensure substantial seed production by self- as well as cross-fertilization. Pollinators may be limited if other more attractive scrub plants bloom at the same time as *B. grandiflora*. Seed herbivory does not appear to be a problem in natural populations of *B. grandiflora*, whereas fungal damage can be in some years; insect damage has also been reported but not observed in this study.

B. grandiflora shows some inbreeding depression in selfed fruits and seeds. However, the degree of inbreeding depression observed in fruits and seeds in this study does not appear to affect the present populations adversely. However, on the basis of other studies on plants, five times more inbreeding depression can be expected throughout the entire life cycle of *B. grandiflora*.

Seed number per fruit was a good predictor of percentage germination; perhaps both variables show the effect of heterosis as controls and cross-pollinated flowers produced the largest number of seeds/capsule. The number of seeds per fruit and seed weight had an inverse relationship. Seed weight was not associated with percentage germination. Plant size was not associated with fruiting success, fruit or seed weight, number of seeds/capsule or percentage germination.

CHAPTER 4
MOLECULAR FINGERPRINTING OF *BONAMIA GRANDIFLORA*

Introduction

Two factors thought to be important in conservation of endangered species are their demography and their genetic richness (Lande, 1988). Whether the limiting factor for the conservation of a species is the number of individuals available or their genetic diversity probably depends on the particular species. In any case, genetic variation is vital for species to survive environmental changes in the short- and long-term (Barret and Kohn, 1991; Frankel and Soulé, 1981; Schaal *et al.*, 1991).

The most popular method to assess genetic diversity has been enzyme electrophoresis. It is a relatively inexpensive method that requires small amounts of sample material, allowing numerous individuals to be sampled; the alleles are inherited in a Mendelian fashion and are co-dominant. On the other hand, enzyme electrophoresis samples a small portion of the genome, silent mutations go unnoticed, and the enzymes used may be subject to selection, as has been demonstrated in *Colias* butterflies (Watt, 1977, 1983), rainbow trout (Leary *et al.*, 1984), *Drosophila melanogaster* (Heinstra *et al.*, 1986), and in European silver fir (Bergmann *et al.*, 1990) among others. In these examples the alleles are not neutral and may lead to erroneous conclusions about the genetic structure of populations.

RFLP (restriction fragment length polymorphisms) and Jeffrey's fingerprinting have also been used to assess genetic identity (Sharon *et al.* 1992; Piper and Parker Rabenold, 1992; Ishii *et al.*, 1993). Both require relatively larger amounts of DNA, are more labor intensive and more costly than enzyme electrophoresis so sample sizes employed are usually small. These two methods, especially RFLP, have been widely used in forensic science and applied plant sciences but are less commonly used in the study of wild species.

With the advent of the polymerase chain reaction (PCR) the direct amplification of DNA fragments became possible (Mullis and Faloona, 1987) and new methods to produce molecular fingerprints of individuals were developed. The RAPD (random amplified polymorphic DNA) method (Welsh and McClelland, 1990; Williams *et al.*, 1990) quickly became popular and has been applied to identify individuals, cultivars, races, and varieties; it has been used in fungal, plant, and animal species. This method is also relatively cheap and requires little material, making the testing of many individuals possible. Another PCR-dependent method of more recent development is AFLP, amplified fragment length polymorphisms (Vos *et al.*, 1995). AFLP is technically more demanding and expensive than RAPD and it also requires more DNA, but it is very reliable and it appears to have higher resolution than either RAPD and or RFLP.

One drawback that RAPD and AFLP share is that these markers behave as dominant. This means that the true number of heterozygotes in populations cannot be assessed directly and population structure must be calculated based on the number of homozygous recessive individuals and the assumption of Hardy-Weinberg equilibrium; obviously this is a serious disadvantage. Although some commercial laboratories claim

to have developed methods that allow the identification of heterozygotic individuals with AFLP based on improved detection of fluorescent tags, no results of these methods appear to have been published.

RAPD, Random Amplified Polymorphic DNA, (Welsh and McClelland, 1990; Williams *et al.*, 1990) is a particular kind of fingerprinting involving DNA amplification directed by oligonucleotide primers of arbitrary sequence. These polymorphic DNA segments are present in some individuals and absent in others, and in this way, they can be used as genetic markers. Several features of this technique make it particularly amenable for studies of population genetics (Williams *et al.*, 1990): it is a relatively fast, cheap, and easy procedure, and it does not need the use of radioactive material or highly sophisticated equipment. It only requires the collection of a small amount of plant material; one leaf of every plant sampled provides enough DNA for use with several different primers. However, this type of marker only reveals presence or absence of bands, so co-dominance cannot be assessed. Although in data analyses these markers are generally treated as loci, in most cases the exact identity of these DNA fragments is not known. It is assumed that co-migrating bands are identical and that markers segregate in a Mendelian fashion. There is some support for these assumptions (Carlson *et al.*, 1991; Dawson *et al.*, 1993; Stammers *et al.*, 1995) but because of the cost and time involved to verify the identity of these DNA fragments, either by sequencing or by using them as probes, it is not possible to do so in most studies.

In studies in which RAPD markers are compared with results from enzyme electrophoresis, RAPDs show more marker diversity (Aagaard *et al.*, 1998; Liu and Furnier, 1993; Mosseler *et al.*, 1992; Peakall *et al.*, 1995). Generally the genetic

structure revealed by both methods agree (Aagaard *et al.*, 1998; Dawson *et al.*, 1993; Haig *et al.*, 1994; Peakall *et al.*, 1995).

In Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995) the genome is digested with restriction enzymes into smaller fragments and some of these fragments are amplified by choosing primers that share part of their sequence with the restriction enzymes. The number and size of bands produced by amplification is highly variable between individuals in the same species. Although relatively new, AFLP has been effective in several species. AFLPs were more efficient than RFLP at detecting polymorphisms in maize (Marsan *et al.*, 1998), rice (Maheswaran *et al.*, 1997), and soybean (Maughan *et al.*, 1996). In *Brassica oleracea* one set of AFLP primers produced 26 markers and thirty-seven RFLP probes detected 66 markers (1:1.8) (Voorrips *et al.*, 1997). AFLP markers were five times more numerous than RAPD markers in potato cyst nematode populations (Folkertsma *et al.*, 1996), and AFLPs were ten times more efficient than RAPDs as markers in cultivated and wild lentil species (Sharma *et al.*, 1996). In soybean, RAPDs detected 0.5 polymorphic loci per primer (Williams *et al.*, 1990), whereas AFLPs revealed approximately eighteen polymorphic loci for each pair of primers (Maughan *et al.*, 1996).

AFLP analysis has been used with wild species such as the symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus* (Mueller *et al.*, 1996), two *Salix* species (Beismann *et al.*, 1997), and endangered species such as *Astragalus cremnophylax* var. *cremnophylax* (Travis *et al.*, 1996).

However, the most frequent use of this technique has been with species of agricultural or silvicultural importance, as a fingerprinting technique in populations, or in the construction of maps and in bulked segregant analysis.

Like RAPD markers, AFLP markers have unknown sequences and co-migrating fragments are assumed to be identical, AFLP markers are also presumed to follow Mendelian segregation. Because AFLP analysis has been used on species of economic importance, which are better understood, and for which more resources are available, we know that these assumptions are true most of the time. For example, a study on potato has shown that 95% of co-migrating bands amplified using the same primers are identical or nearly so (Rouppe van der Voort *et al.*, 1997). Mendelian segregation has been found in AFLP markers in cattle (Ajmone Marsan *et al.*, 1997), in 91.6% of the AFLP markers seen in *Pinus sylvestris* (Lerceteau and Szmidt, (submitted), in 78% of markers in rice (Maheswaran *et al.*, 1997), and in 94% of the markers in *Picea abies* (Paglia and Morgante, 1998).

In RAPD and AFLP fingerprinting it is assumed that each DNA fragment or band scored represents a locus and its presence or absence represent the two allelic forms due to mutations leading to the lack of restriction or amplification at selected sites. However, large insertions can also result in the lack of amplification products because DNA polymerase can only amplify fragments that are approximately 5 000 base pairs. Another problem that arises with this assumption is that deletions or insertions between the primers can result in a fragment with different weight and therefore gel migration distance. These co-dominant loci have been identified in some studies. For example in *Picea abies* 14% of the markers were co-dominant (Paglia and Morgante, 1998). Out of

six markers found to segregate in a Mendelian fashion, five were dominant and one was co-dominant in soybean (Maughan *et al.*, 1996), in three barley populations co-dominant markers ranged from 6% to 12.6% (Waugh *et al.*, 1997), and in rice 10.6% of the AFLP markers were co-dominant (Maheswaran *et al.*, 1997).

Other types of inheritance seem rarer than co-dominance. In a soybean study one marker was found to have maternal inheritance, the other thirty-nine segregated as dominant Mendelian markers (Prabhu and Gresshoff, 1994).

Ideally, molecular markers should be distributed throughout the genome, especially for mapping applications. AFLPs have been found to be unevenly distributed and clustered around the centromeres in barley (Qi *et al.*, 1998) and *Arabidopsis thaliana* (Alonso-Blanco *et al.*, 1998) but they were scattered throughout the genome in rice (Zhu *et al.*, 1998) (Maheswaran *et al.*, 1997).

In this study it will be assumed that co-migrating bands are identical; that there are only two alleles for each locus, a dominant (amplified) and a recessive (absent, null); that the populations are in Hardy-Weinberg equilibrium; and that segregation follows Mendelian rules.

Three methods of identification of individuals were used in *B. grandiflora* because very little variation was detected either with enzyme electrophoresis or with RAPD markers. As mentioned before, enzyme electrophoresis is cheaper than the other two techniques, it uses small amounts of tissue, and it produces co-dominant markers so it was the method selected in the first attempt to characterize genetic variation in *B. grandiflora* populations. When results became available, it was apparent that isozyme electrophoresis would not produce enough marker diversity to resolve population

structure and RAPD markers were employed. This second method also yielded little genetic diversity information and therefore a new, more discriminating technique, AFLP was finally employed. The reason for not employing directly AFLP markers in the first sampling is that the method had not been developed at the time. AFLP is also more expensive, it requires the use of radioactive materials or expensive fluorescent tags and it requires the use of more tissue than either enzyme electrophoresis or RAPD.

The sampling was done in stages because the primary objective was to assess the genetic diversity in the Ocala National Forest, to facilitate the management of this species within this area. When little genetic diversity was encountered, the logical next step was to assess whether this low genetic diversity was only found in the Ocala National Forest or if it was a feature of this species as a whole.

The objectives of the study are the following: 1- to detect and measure genetic variation as expressed by isozymes, RAPDs, and AFLPs in *Bonamia grandiflora*. 2- to determine the genetic relationships between *B. grandiflora* wild populations. Given the results of the phenotypic characterization of *B. grandiflora* individuals and because this species is a rare endemic and peripheral in the distribution of the genus (Myint, 1966), reduced levels of genetic variation were expected.

Methods

Estimation of Genetic Variability Within the Ocala National Forest Local Populations by Enzyme Electrophoresis

A leaf was collected from each of 54 individual plants growing in the Seed Orchard of the Forest Service located in the Ocala National Forest. The plants in the orchard belong to seed lot # 0; the seeds that produced those plants were collected by Forest Service personnel throughout Ocala National Forest local populations. The leaves were transported on ice and kept refrigerated until they were processed. The initial testing was done at the request of the Forest Service to assess the genetic diversity of their *Bonamia grandiflora* collection, prior to possible re-introduction of the species into sites from which it had been lost.

A Tris-HCl grinding buffer/PVP solution was used to extract enzymes from the leaf tissues (Gottlieb, 1981; Soltis *et al.*, 1983). The resulting mixture was centrifuged at 10,000 rpm for 2 min. in an Eppendorf centrifuge (Model 5415); the supernatant was collected and used to imbibe wicks of Whatman #1 filter paper. The wicks were inserted in agarose gels (1%) in BioRad SubCell electrophoretic apparatus. Gels were stained for alcohol dehydrogenase (ADH), aspartate aminotransferase (AAT), glucose-6-phosphate isomerase (GPI), isocitrate dehydrogenase (IDH), and phosphoglucomutase (PGM) using specific stains (Murphy *et al.*, 1990). The best resolved bands resulted from System 5 (Soltis *et al.*, 1983) for ADH, GPI, and PGM and System 3 (Soltis *et al.*, 1983) for AAT and IDH.

All solutions were ice cold and gels were kept cool by putting ice packs on and under the electrophoresis apparatus and placement in a cold room. Electrophoresis took 4 to 6 h. at 65 mA.

The mean number of alleles per locus (A), the percentage of polymorphic loci (P), the expected heterozygosity (He), and the fixation index or inbreeding coefficient (F) (Wright, 1984) were calculated. Hardy-Weinberg equilibrium was assessed with a χ^2 test.

Estimation of Genetic Variability Within and Between Ocala National Forest Local Populations and Other Florida Populations by RAPD

In this study, there were two stages in the sampling; firstly, individuals from the Ocala National Forest Seed Orchard were sampled and tested. These plants originated from seeds collected from 17 sites throughout the forest by the Forest Service personnel. In the second stage, individuals from throughout the geographic range of the species were collected and analyzed. The populations sampled were the following: Ocala National Forest North and South of Highway 40, Fenton Road, Hull Road, Bok Tower Garden collection, Tower, and The Nature Conservancy preserves in Saddleblanket Lakes, Tiger Creek, and Sun Ray (Fig. 4-1).

DNA was isolated from fresh leaf tissue (Ausubel *et al.*, 1993). *B. grandiflora* leaf tissues contain mucilaginous compounds that can make PCR difficult. To remove the polysaccharides, after extraction the DNA was precipitated with ethanol in a 2 M Na Cl

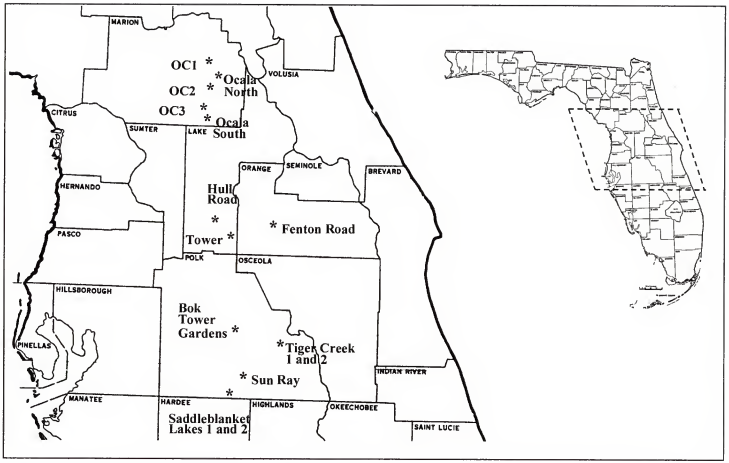


Figure 4-1. Populations of *Bonamia grandiflora* that were sampled in the genetic fingerprinting of this species. All of them are located on the Central Ridge of the Florida peninsula.

solution (Fang *et al.*, 1992) and suspended in TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

The amplification reactions were done in 25 µl of PCR mixture, according to the Operon 10-mer kit instructions (Operon Technologies, Inc.). The PCR mixture was prepared with 10x PCR buffer Magnesium-free (Promega), 25mM $MgCl_2$ (Promega), 100 µM each of dATP, dTTP, dCTP, and dGTP (Promega), 0.5 U of Taq polymerase (Promega), and glass-distilled, autoclaved water. Approximately 80 ng of total DNA were added to the amplification reaction as a template. For each RAPD primer, preliminary work indicated the optimum concentration of $MgCl_2$ and annealing temperature that produced the least band ladders and other spurious bands. The amplification temperatures for the 40 cycles in a Coy Thermocycler were 94°C for denaturation, 40°C (or higher) for annealing, and 72°C for extension.

Two primer kits, Operon kit A and R of 10-base oligonucleotides (Operon Technologies, Inc.) were used. These are synthetic primers of randomly chosen sequences, their (G+C) content is 60 to 70%, and they have no self-complimentary ends. The two 20-primer kits were tested to identify primers that rendered the clearest amplification products. Fourteen RAPD primers and a combination of two 20-base long chloroplast universal primers, E and F (Taberlet *et al.*, 1991) were used with *B. grandiflora* samples (Table 4-1). The two chloroplast universal primers belong to a set of six primers designed to amplify non-coding and highly variable regions of chloroplast DNA (Taberlet *et al.*, 1991). The primers target conserved regions that flank the

variable non-coding regions and enable the use of these primers in a variety of plant species (Taberlet *et al.*, 1991).

Table 4-1. Sequences of primers used in the amplification of *Bonamia grandiflora* samples. The first 15 primers belong to Operon Kits A and R (Operon Technologies); the last two primers were synthesized to amplify non-coding regions of chloroplast DNA (Taberlet *et al.*, 1991).

Primer name	Primer sequence (5' to 3')
OPA-1	CAGGCCCTTC
OPA-3	AGTCAGCCAC
OPA-7	GAAACGGGTG
OPA-9	GGGTAACGCC
OPA-15	TTCCGAACCC
OPA-17	GACCGCTTGT
OPA-20	GTTGCGATCC
OPR-2	CACAGCTGCC
OPR-6	GTCTACGGCA
OPR-8	CCCGTTGCCT
OPR-10	CCATTCCCCA
OPR-11	GTAGCCGTCT
OPR-12	ACAGGTGCGT
OPR-13	GGACGACAAG
OPR-16	CTCTGCGCGT
Chloroplast universal primer E	GGTTCAAGTCCCTCTATCCC
Chloroplast universal primer F	ATTTGAACTGGTGACACGAG

To monitor for DNA cross contamination, negative controls with no template DNA were included in each batch of samples amplified. The PCR products were run in either 1.4% (Ocala samples) or 1.8 % (mixed population samples) agarose gels and stained in an ethidium bromide solution. They were observed and photographed under UV light. In the Ocala National Forest photographs, one of the samples was included as a standard. A molecular marker, ϕX174/Hae III (Promega), was included in photographs of samples of mixed populations. If a sample showed a novel band, its amplification was repeated

to assess reproducibility of such band. It has been demonstrated that the error rate in scoring RAPD bands increases when such bands are faintly stained (Weeden *et al.*, 1992). Therefore, a locus was included in the study only if the band was clear and could be scored unambiguously. A locus was considered polymorphic if the presence and absence of a band were observed; monomorphic when it was present in all the individuals.

Estimation of Genetic Variability Within and Between Florida Populations by AFLP Fingerprinting

DNA Isolation and AFLP Reactions

Samples from ten local populations or subpopulations located in six populations were collected (Fig. 4-1). The collection schedule and abbreviations used in tables and figures are the following:

Population	Subpopulations
Sun Ray (SR)	
Saddleblanket Lakes (SB)	SB1, SB 2
Tiger Creek (TC)	TC 1, TC 2
Fenton Road (F)	
Hull Road (H)	
Ocala National Forest (OC)	OC 1, OC 2, OC 3

According to the size of the plant, one to six leaves were collected from each individual. They were preserved in liquid nitrogen during their collection and transport

and kept frozen at –70 °C until they were extracted. Total DNA was isolated with double phenol extractions (Ausubel *et al.*, 1993); the samples were exposed to RNAse for a day after the first phenol extraction. Because large amounts of mucilaginous substances were still present in the samples, they were precipitated again with CTAB. Finally, the DNA was suspended in TE.

The original AFLP protocol was followed (Vos *et al.*, 1995); it consists of the digestion of total DNA with two restriction enzymes, a rare and a frequent cutter. Then the DNA fragments are ligated to two adapters that have sequences corresponding to the two restriction enzymes. These ligated fragments are amplified twice. First is the preamplification PCR (Vos *et al.*, 1995), which uses two primers that correspond to the sequences of the two enzymes and adapters and one extra base to select out fifteen out of the sixteen classes of DNA fragments. The second PCR uses two primers that have the same sequences as in the preamplification but they have three extra bases to further select out DNA fragments; one of the primers (*Eco*RI, in this case) is also radioactively labeled to allow the visualization of the resulting banding profile on the polyacrylamide gels.

Adapters and Primers

The adapters and primers were constructed as shown in the original protocol (Vos *et al.*, 1995) and they were synthesized by Genosys. The sequences are the following:

*Eco*RI adapter 5'-CTCGTAGACTGCGTACC

CATCTGACGCATGGTTAA-5'

*Mse*I adapter	5'-GACGATGAGTCCTGAG
	TACTCAGGACTCAT-5'
*Eco*RI+1 primer	5'-GACTGCGTACCAATTCC
*Eco*RI+3 primer	5'-GACTGCGTACCAATTCCAC
*Mse*I+1 primer	5'-GATGAGTCCTGAGTAAC
*Mse*I+3 primer	5'-GATGAGTCCTGAGTAACGA

For each sample one fourth of a microgram of total DNA was digested with 2.5 U of *Eco*RI (Promega) and 2.5 U of *Mse*I (Gibco BRL) in REACT 1 buffer (Gibco BRL) in a total volume of 20 µl for 3 h at 37° C. The digested DNA was ligated with 2.5 pMol of the *Eco*RI adapter and 25 pMol of the *Mse*I adapter. The ligation reaction proceeded overnight at room temperature and it was done with 0.5 U of T4 DNA ligase (Promega) and its corresponding Ligase buffer (Promega).

The DNA-adapters solution was diluted to 250 µl (1:10) with TE. Preamplification was done with 5 µl of the DNA-adapters solution, 30 ng of the *Eco*RI+1 primer, 30 ng of the *Mse*I+1 primer, 0.4 U of *Taq* DNA Polymerase (Promega), Reaction buffer (Promega) and 1.5 mM $MgCl_2$. The 40 cycles of amplification were done at 94° C (30 s) for denaturation, 56° C (1 min) for annealing, and 72° C (1 min) for extension.

The second amplification was done with 30 ng of *Mse*I+3 primer and 5 ng of *Eco*RI+3 primer. The latter was 5'-end labeled as follows: 150 µCi [γ^{33}P] ATP, 10 U of

T4 (Promega), Kinase buffer (Promega) and 1μg of *Eco*RI+3 primer, in a total volume of 20 μl were incubated for half hour at 37° C and then purified with a NucTrap Probe Purification column (Stratagene). The preamplification reaction products were diluted 10-fold and 5 μl were used as the DNA template for the amplification. The amplification reactions were done at the same temperatures and for the same duration as before but the number of cycles was 36 instead of 40.

The amplification products were run in a denaturing 8% polyacrylamide gel at 40V together with pGEM DNA molecular marker (Promega). The pGEM marker was 5'-end labeled with [γ^{32}P] ATP as described for the primers but the marker was previously dephosphorylated with alkaline phosphatase (Promega).

Band Scoring

Autoradiographs of the AFLP banding profiles were quantified using an IS1000 Digital Imaging System (Alpha Innothech Corp.). The Imaging System quantifies the following: the distance of the band to the gel well, band width, height, area, and percentage of the total intensity of the band for that lane. The latter was used to decide which bands were scored as present and which were absent; an effort was made to maximize the inclusion of meaningful information but to exclude the numerous short artifactual PCR products.

Data Analysis

Because of the nature of AFLP markers, several points need to be considered. The band patterns are not true genotypes, they are phenotypes; as is the case in enzyme

electrophoresis and RAPD markers we do not know the exact sequence of the markers and some mutations may go unnoticed. However, just as for isozymes and RAPDs, in the analyses AFLP markers will be treated as genotypes. AFLP markers are dominant so the true frequency of heterozygotic individuals is unknown. The AFLP banding profiles of 10 subpopulations in 6 population of *B. grandiflora* were analyzed with four programs, POPGENE 1.21 (Yeh *et al.*, 1997), AMOVA 1.55 (Excoffier, 1995), MINSPNET (Excoffier, 1993), and the PHYLIP package of programs (Felsenstein, 1993).

A phylogenetic tree was constructed with the PHYLIP package. PENNY, a program that estimates phylogenies for discrete character data, was used to generate thousands of trees. The best hundred phylogenies produced by PENNY were used as the input for CONSENSE, a program that produces consensus trees by the majority-rule criterion. PENNY constructs the most parsimonious trees by the "branch and bound" algorithm and it is not sensitive to input order. The phylogenetic trees were drawn with the aid of TreeView (Page, 1997).

The same distance matrix was used in AMOVA and MINSPNET. A matrix was constructed with the band scoring for each individual. The presence of a band was scored as a one, its absence as a zero. Based on this matrix a distance matrix was constructed by pairwise comparisons of each distinct individual to itself and every other individual. This was an Euclidean phenetic distance matrix based on the count of the number of band presence differences between individuals (Excoffier *et al.*, 1992). AMOVA allows the visualization of how the variability is organized at the population and group or regional level. This method was originally developed for the analysis of

mitochondrial DNA haplotypes (Excoffier *et al.*, 1992) but it appears that it can be directly applied to the analysis of RAPD and AFLP phenotypes (Huff *et al.* 1993, Haig *et al.* 1994, Travis *et al.* 1996, Scott *et al.* 1997). It performs the analysis of population structure at the molecular level by computing molecular variance components (Excoffier, 1995). The significance of the observed variance components is tested by simulations in which individuals are randomly reallocated into subpopulations and populations and the null distribution of each variance component is estimated from a number of independent reiterations (Haig *et al.* 1994); in this study the number of iterations was 700. If the species is haploid or completely selfing, AMOVA can also compute analogs of the F-statistics, population pairwise F_{ST}, and coancestry coefficients but in the case of phenetic matrices such as with RAPD of AFLP markers, it is not directly possible to obtain these estimates.

MINSPNET constructs minimum spanning networks (MSN) that minimize the total number of mutational events and therefore the length of the network, and the population molecular variance expressed both as molecular differences and allele frequencies (Excoffier and Smouse, 1994). The distance matrix can be modified to incorporate geographic information, however, this was not done in the present analysis because the interest was in finding out whether the genetic structure reflected the geographic relationships of the populations.

POPGENE calculates genetic distances and genetic identity and an UPGMA tree based on genetic distances for both Nei's 1972 (Nei, 1972) and 1978 indices (Nei, 1978). It also calculates genotypic frequencies, allelic frequencies, allele number, effective allele number, expected heterozygosity, the Shannon information index (Shannon and

Weaver, 1949) and F-statistics, gene flow, homogeneity tests, both χ^2 and G tests, and tests for Hardy-Weinberg equilibrium if co-dominant markers are used. The analyses can be done with individuals as members of a single population or multiple populations or populations that belong to different groups (subpopulations of populations). The program can also use co-dominant or dominant markers. As mentioned above, both RAPD and AFLP markers are dominant and this prevents the direct assessment of the frequency of heterozygotic individuals. Therefore, to calculate F-statistics, Hardy-Weinberg equilibrium was assumed. However, simulations were made assuming disequilibrium in all the subpopulations; the results are discussed.

Besides AMOVA, another way to assess the distribution of genetic variation is to partition the total diversity (H_t) into a within population diversity component (H_s) and a between population component (D_{st}); $H_t = H_s + D_{st}$ (Nei, 1977). The coefficient of gene differentiation is defined as follows: $G_{st} = D_{st}/ H_t$ (Nei, 1977). POPGENE was used to calculate H_t, H_s, and G_{st} for the forty-six loci.

The UPGMA (unweighted pair group method using arithmetic average) dendrogram produced by POPGENE 1.21 is a modification of the program NEIGHBOR of PHYLIP 3.5c. A genus closely related to *Bonamia*, *Stylisma* (see Chapter 1)was used as a reference in the construction of the UPGMA tree. In the analyses done by POPGENE 1.21 the distance matrix used was based on Nei's unbiased genetic distance (Nei, 1978). In Nei's unbiased genetic distance divergence occurs due to genetic drift and mutation and distance is proportional to the time since divergence (Nei, 1978).

To assess whether genetic relationships between populations reflected geographic patterns, another UPGMA dendrogram for 10 populations of *Bonamia grandiflora* was constructed manually. The distances are the approximated shortest distances between pairs of populations and were measured on maps.

The homogeneity of frequency of bands between the nine populations was tested with G-tests performed by POPGENE 1.21 (Yeh *et al.*, 1997); this program also calculated Nei's unbiased measures of genetic identity and genetic distance (Nei, 1978). The percentage of polymorphic loci was calculated manually from the band matrix for each individual. A band was considered polymorphic if its presence and absence were observed and monomorphic if it was present in all the individuals.

Results

Estimation of Genetic Variability Within the Ocala National Forest Local Populations by Enzyme Electrophoresis

Four out of the five enzymes were monomorphic (AAT, ADH, IDH, and PGM). PGM showed a second locus but it was too poorly resolved to be scored (Fig. 4-2). The zymograms of AAT, ADH, and IDH were similar to the one of PGM, but with only one locus and have not been reproduced. GPI was polymorphic; four individuals had a slow allele, the other 50 had the fast allele (Fig. 4-2). A second locus was too poorly resolved to be scored. Two individuals, 54 and 55 did not give allelic products at the expected positions when the gels were stained to detect GPI, PGM, and AAT (Fig. 4-1). Because these gels were run after the ones to detect ADH and IDH, it is suspected that enzymes had degraded in those samples.

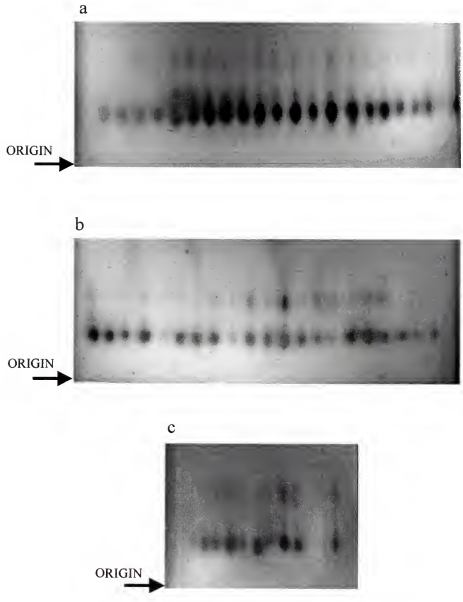
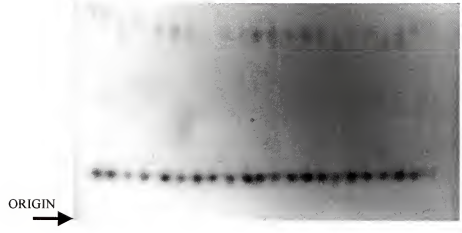


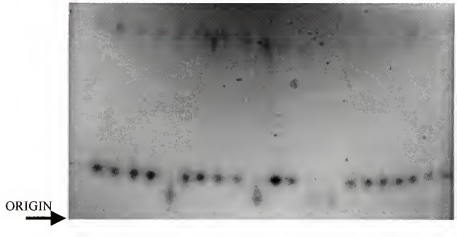
Figure 4-2. Zymograms of glucose-6-phosphate isomerase (GPI) and phosphoglucomutase (PGM) from *Bonamia grandiflora.*
a) Individuals 1 to 22 stained to detect PGM.
b) Individuals 23 to 43 stained to detect PGM.
c) Individuals 44 to 56 stained to detect PGM.

Figure 4-2—continued.

d



e



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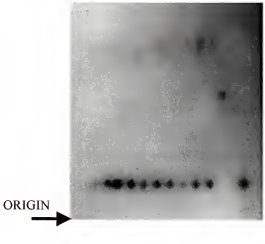


The mean number of alleles per locus, A, was 1.2. The percentage of polymorphic loci, P, was 20%. There were no heterozygous individuals for any of the enzymes and so Ho = 0 for all and He = 0.13 for GPI and 0 for the other four enzymes. For GPI F = 1.00. The population was not in Hardy-Weinberg equilibrium ($\chi^2 = 58.82$, $p < 0.001$).

Estimation of Genetic Variability Within and Between Ocala National Forest Local Populations and Other Florida Populations by RAPD

Despite the optimization of annealing temperature, $MgCl_2$ concentration, and the standardization of amount of DNA template in the amplification reactions, there were differences in the intensity of the markers between samples. Preliminary testing of different concentrations of primers, dNTPs, and Taq polymerase did not give better results than the final protocol described in the methods. The time between denaturing of the DNA and annealing of the primer to the template appeared very important in preliminary trials with *B. grandiflora* samples. When a Robocycler was used practically no amplification products appeared; this thermocycler had four heating/cooling blocks that kept four constant temperatures; when the samples were moved from block to block the shift from denaturing to annealing temperatures was, therefore, almost instantaneous. This effect on amplification products in RAPDs analysis has also been noted in soybean samples (Schweder *et al.*, 1995) in which more products were amplified when longer times were allowed between denaturing and annealing.

The amplification products of the Ocala National Forest samples can be seen in Fig. 4-3 to 4-8. A locus was included in the study only if the band was clear in all the individuals and could be scored unambiguously, and when it appeared in the two

amplifications of each individual sample. Twenty-three markers were amplified with six RAPD primers. Naturally, the banding profiles differed between primers, given that the amplified regions of DNA are different and result in DNA fragments of different lengths and therefore different gel migration distance. However, for all primers used the individuals sampled were essentially monomorphic. In other words, for the sequences tested with RAPD markers the individuals showed very few differences between or within the local Ocala subpopulations. A few individuals showed novel bands, for example individuals FS39 and FS45 amplified with primer OPA-17 (Fig. 4-5) or lacked a band such as FS20 with primer OPR-6 (Fig. 4-7).

Samples from throughout the geographic range of *B. grandiflora* were also homogeneous when fingerprinted with RAPD and chloroplast universal primers E/F (Fig. 4-9 to 4-22). A total of 44 markers were amplified. The reproducibility of bands became substantially more difficult when individuals from different populations were used. The amount of mucilaginous compounds was variable and these and perhaps other secondary compounds made amplification difficult.

Estimation of Genetic Variability Within and Between Florida Populations by AFLP Fingerprinting

AFLP was a much more efficient technique for detecting variation in *B. grandiflora* than RAPD. Forty-six bands were scored and analyzed (Fig. 4-23 to 4-29); these bands ranged between 100 and 500 bases long. A few of the samples were replicated throughout the entire fingerprinting protocol. The results of the replicated

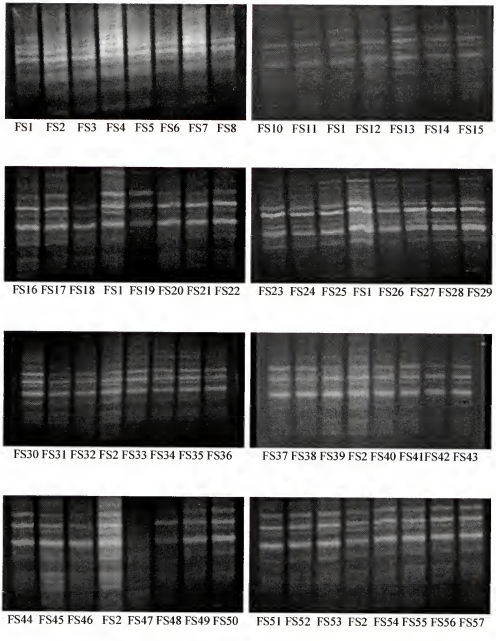

FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8
FS10 FS11 FS1 FS12 FS13 FS14 FS15
FS16 FS17 FS18 FS1 FS19 FS20 FS21 FS22
FS23 FS24 FS25 FS1 FS26 FS27 FS28 FS29
FS30 FS31 FS32 FS2 FS33 FS34 FS35 FS36
FS37 FS38 FS39 FS2 FS40 FS41FS42 FS43
FS44 FS45 FS46 FS2 FS47 FS48 FS49 FS50
FS51 FS52 FS53 FS2 FS54 FS55 FS56 FS57

Figure 4-3. Amplification products of *Bonamia grandiflora* with RAPD primer OPA-3.
The individuals belong to several populations of the Ocala National Forest. Individuals FS1 or FS2 were included in all the photographs as controls.

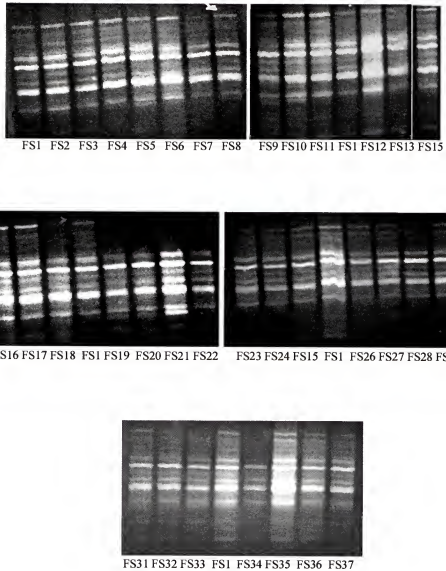

FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8
FS9 FS10 FS11 FS1 FS12 FS13 FS15
FS16 FS17 FS18 FS1 FS19 FS20 FS21 FS22
FS23 FS24 FS15 FS1 FS26 FS27 FS28 FS29
FS31 FS32 FS33 FS1 FS34 FS35 FS36 FS37

Figure 4-4. Amplification products of *Bonamia grandiflora* with RAPD primer OPA-7.
The individuals belong to several populations of the Ocala National Forest. Individuals FS1 or FS2 were included in all the photographs as controls. C = negative control; no template was added to the amplification reaction.

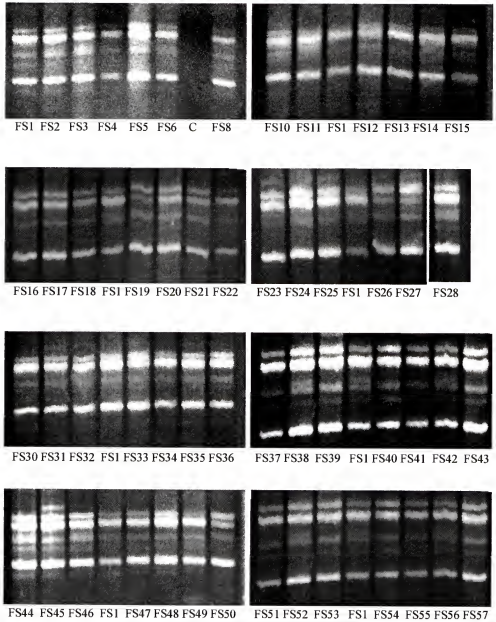

FS1 FS2 FS3 FS4 FS5 FS6 C FS8
FS10 FS11 FS1 FS12 FS13 FS14 FS15
FS16 FS17 FS18 FS1 FS19 FS20 FS21 FS22
FS23 FS24 FS25 FS1 FS26 FS27 FS28
FS30 FS31 FS32 FS1 FS33 FS34 FS35 FS36
FS37 FS38 FS39 FS1 FS40 FS41 FS42 FS43
FS44 FS45 FS46 FS1 FS47 FS48 FS49 FS50
FS51 FS52 FS53 FS1 FS54 FS55 FS56 FS57

Figure 4-5. Amplification products of *Bonamia grandiflora* with RAPD primer OPA-17.
The individuals belong to several populations of the Ocala National Forest. Individuals FS1 or FS2 were included in all the photographs as controls. C = negative control; no template was added to the amplification reaction.

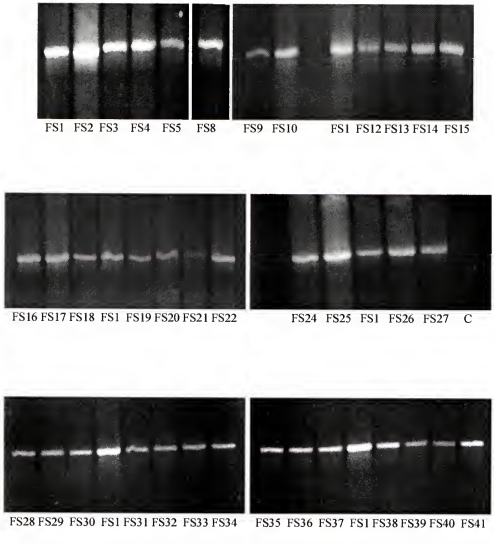
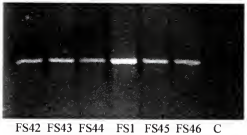
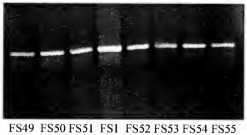

FS1 FS2 FS3 FS4 FS5 FS8 FS9 FS10 FS1 FS12 FS13 FS14 FS15
FS16 FS17 FS18 FS1 FS19 FS20 FS21 FS22 FS24 FS25 FS1 FS26 FS27 C
FS28 FS29 FS30 FS1 FS31 FS32 FS33 FS34 FS35 FS36 FS37 FS1 FS38 FS39 FS40 FS41

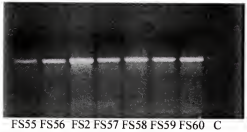
Figure 4-6. Amplification products of *Bonamia grandiflora* with RAPD primer OPR-2.
The individuals belong to several populations of the Ocala National Forest. Individuals FS1 or FS2 were included in all the photographs as controls. C = negative control; no template was added to the amplification reaction.



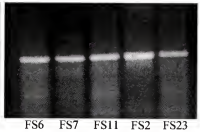
FS42 FS43 FS44 FS1 FS45 FS46 C



FS49 FS50 FS51 FS1 FS52 FS53 FS54 FS55

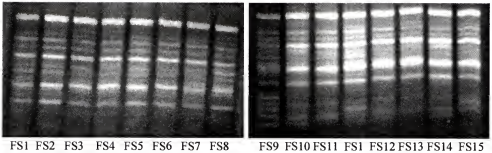


FS55 FS56 FS2 FS57 FS58 FS59 FS60 C



FS6 FS7 FS11 FS2 FS23

Figure 4-6--continued.



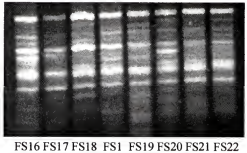


Figure 4-7. Amplification products of *Bonamia grandiflora* with RAPD primer OPR-6.
The individuals belong to several populations of the Ocala National Forest. Individuals FS1 or FS2 were included in all the photographs as controls.

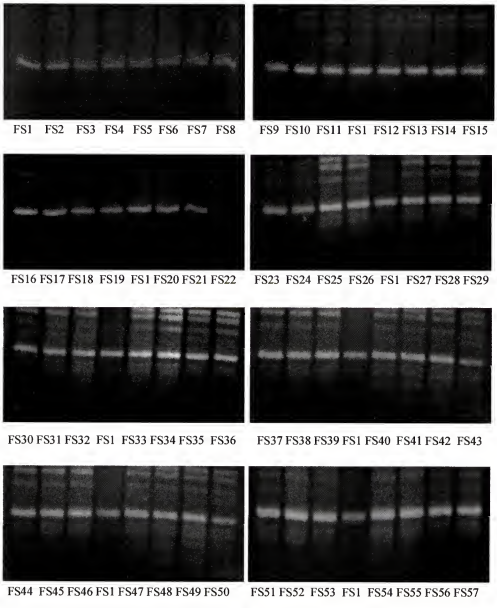

FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8
FS9 FS10 FS11 FS1 FS12 FS13 FS14 FS15
FS16 FS17 FS18 FS19 FS1 FS20 FS21 FS22
FS23 FS24 FS25 FS26 FS1 FS27 FS28 FS29
FS30 FS31 FS32 FS1 FS33 FS34 FS35 FS36
FS37 FS38 FS39 FS1 FS40 FS41 FS42 FS43
FS44 FS45 FS46 FS1 FS47 FS48 FS49 FS50
FS51 FS52 FS53 FS1 FS54 FS55 FS56 FS57

Figure 4-8. Amplification products of *Bonamia grandiflora* with RAPD primer OPR-16.
The individuals belong to several populations of the Ocala National Forest. Individuals FS1 or FS2 were included in all the photographs as controls.

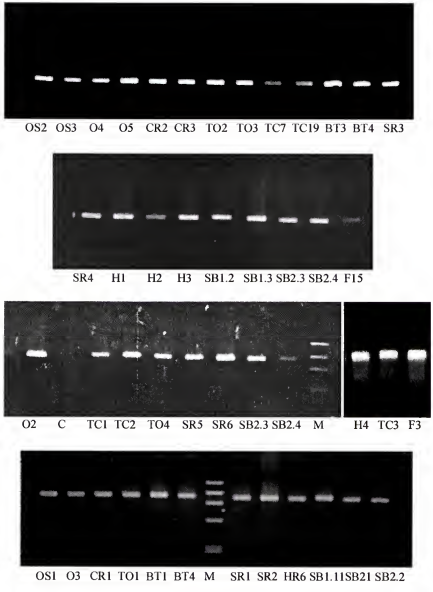

OS2 OS3 O4 O5 CR2 CR3 TO2 TO3 TC7 TC19 BT3 BT4 SR3
SR4 H1 H2 H3 SB1.2 SB1.3 SB2.3 SB2.4 F15
O2 C TC1 TC2 TO4 SR5 SR6 SB2.3 SB2.4 M H4 TC3 F3
OS1 O3 CR1 TO1 BT1 BT4 M SR1 SR2 HR6 SB1.11SB21 SB2.2

Figure 4-9. Amplification products of *Bonamia grandiflora* with chloroplast Universal primers E/F.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. C = negative control; no template was added to the amplification reaction. M = Ø X 174/Hae III molecular marker.

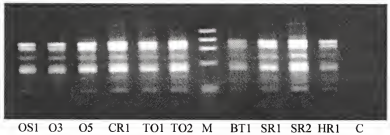


Figure 4-10 . Amplification products of *Bonamia grandiflora* with RAPD primer OPA-1.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. C = negative control; no template was added to the amplification reaction. M = Ø X 174/Hae IIImolecular marker.

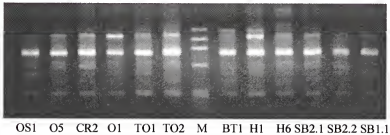


Figure 4-11 . Amplification products of *Bonamia grandiflora* with RAPD primer OPA-7.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. C = negative control; no template was added to the amplification reaction. M = Ø X 174/Hae III molecular marker.

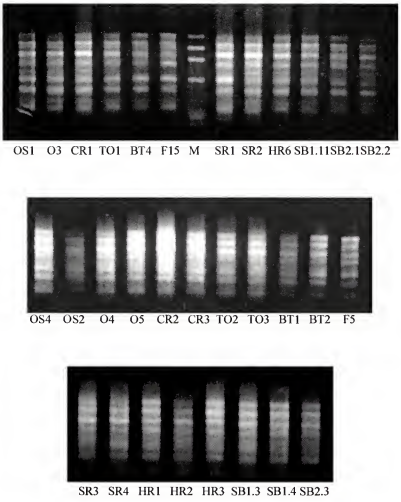


Figure 4-12. Amplification products of *Bonamia grandiflora* with RAPD primer OPA-3.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site.
M = Ø X 174/Hae III molecular marker.

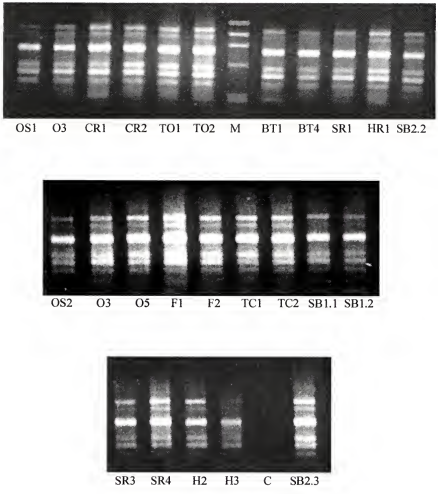


Figure 4-13. Amplification products of *Bonamia grandiflora* with RAPD primer OPA-9.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. C = negative control; no template was added to the amplification reaction. M = Ø X 174/Hae III molecular marker.

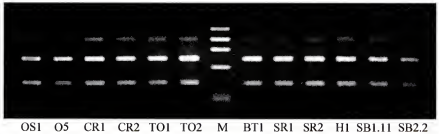


Figure 4-14 . Amplification products of *Bonamia grandiflora* with RAPD primer OPA-15.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site.
M = Ø X 174/Hae III molecular marker.

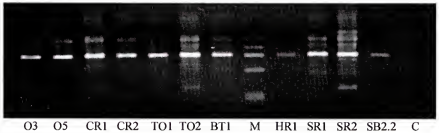
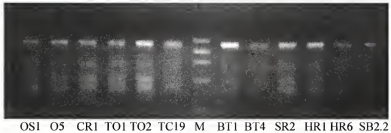
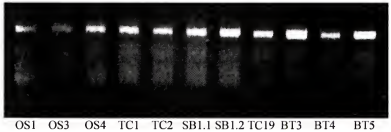


Figure 4-15. Amplification products of *Bonamia grandiflora* with RAPD primer OPA-17.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. C = negative control; no template was added to the amplification reaction.
M = Ø X 174/Hae III molecular marker.





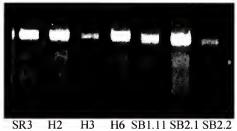


Figure 4-16 . Amplification products of *Bonamia grandiflora* with RAPD primer OPR-2.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. M = Ø X 174/Hae III molecular marker

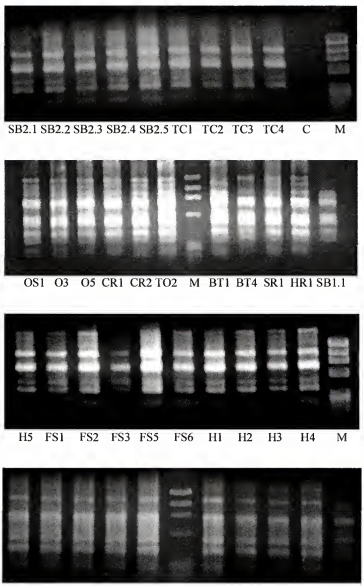

SB2.1 SB2.2 SB2.3 SB2.4 SB2.5 TC1 TC2 TC3 TC4 C M
OS1 O3 O5 CR1 CR2 TO2 M BT1 BT4 SR1 HR1 SB1.1
H5 FS1 FS2 FS3 FS5 FS6 H1 H2 H3 H4 M
SR1 SR2 SR3 SR4 SR5 M SB1.2 SB1.3 SB1.4 SB2.5 SB2.6

Figure 4-17. Amplification products of *Bonamia grandiflora* with RAPD primer OPR-8.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, FS = Forest Service samples, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. C = negative control; no template was added to the amplification reaction. M = Ø X 174/Hae III molecular marker.

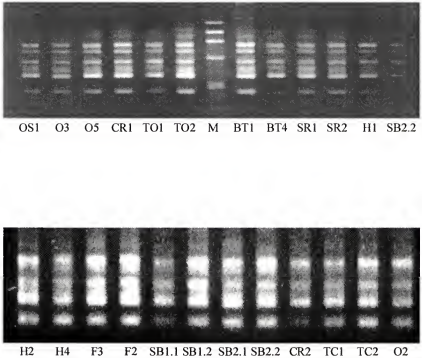


Figure 4-18. Amplification products of *Bonamia grandiflora* with RAPD primer OPR-10.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, FS = Forest Service samples, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. M = Ø X 174/Hae III molecular marker.

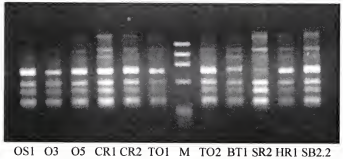


Figure 4-19. Amplification products of *Bonamia grandiflora* with RAPD primer OPR-11.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, FS = Forest Service samples, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. M = Ø X 174/Hae III molecular marker.

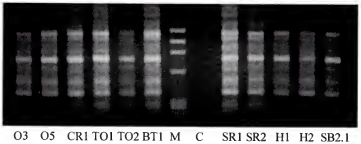
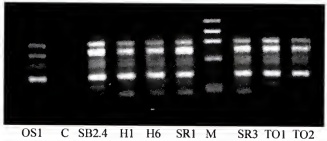


Figure 4-20. Amplification products of *Bonamia grandiflora* with RAPD primer OPR-12.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, FS = Forest Service samples, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. C = negative control; no template was added to the amplification reaction. M = Ø X 174/Hae III molecular marker.



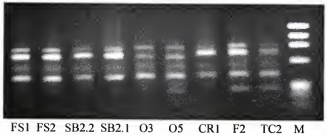
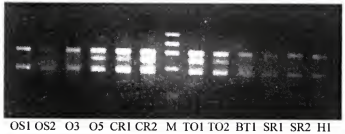


Figure 4-21. Amplification products of *Bonamia grandiflora* with RAPD primer OPR-13.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, FS = Forest Service samples, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. C = negative control; no template was added to the amplification reaction. M = Ø X 174/Hae III molecular marker.



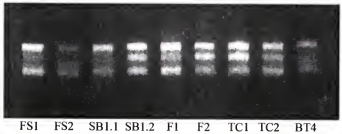


Figure 4-22. Amplification products of *Bonamia grandiflora* with RAPD primer OPR-16.
The individuals belong to several populations on the central ridge of the Florida peninsula . BT = Bok Tower Garden collection, CR = Davenport Creek, F = Fenton Road, FS = Forest Service samples, H = Hull Road, O = Ocala northern population, OS = Ocala southern population, SB = Saddleblanket Lakes Preserve, SR = Sun Ray Preserve, TC = Tiger Creek Preserve, TOW = Tower Site. M = Ø X 174/Hae III molecular marker.

Figure 4-23. Autoradiograph of AFLP from *Bonamia grandiflora* generated using the primer combination *Mse*I-CGA/*Eco*RI-CAC. All the samples were collected in the Ocala National Forest. 1 = individuals from subpopulation number one. 3 = individuals from subpopulation number three. Arrows indicate bands described in the text.

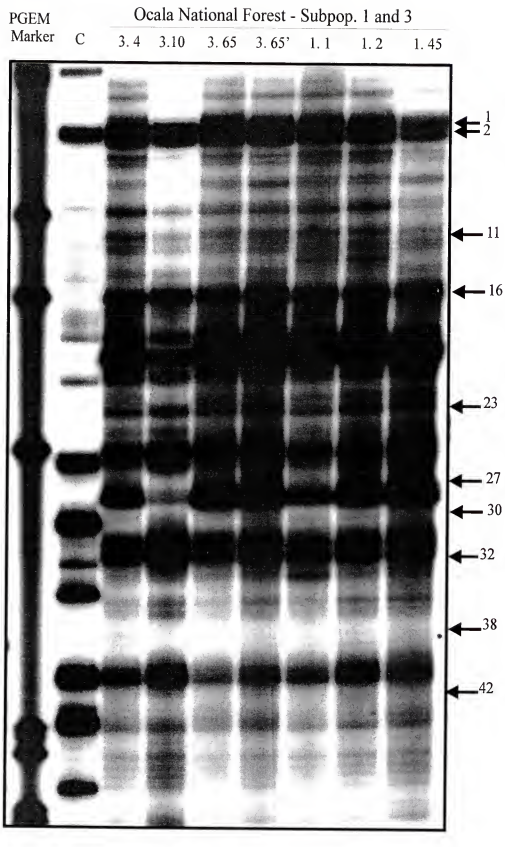

PGEM
Marker
C
Ocala National Forest - Subpop. 1 and 3
3. 4
3.10
3. 65
3. 65'
1. 1
1. 2
1. 45
1
2
11
16
23
27
30
32
38
42

Figure 4-24. Autoradiograph of AFLP from *Bonamia grandiflora* generated using the primer combination *Mse*I-CGA/*Eco*RI-CAC. All the samples were collected in the Ocala National Forest. 2 = individuals from subpopulation number two.

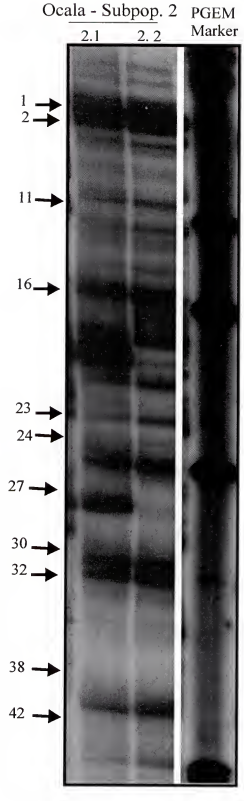

Ocala - Subpop. 2
2.1
2. 2
PGEM
Marker
1
2
11
16
23
24
27
30
32
38
42

Figure 4-25. Autoradiograph of AFLP from *Bonamia grandiflora* generated using the primer combination Mse I-CGA/EcoRI-CAC. Samples were collected in the Ocala National Forest subpopulation number two, and two subpopulations in theTiger Creek Preserve. 1 = individuals from subpopulation number one. 2 = individuals from subpopulation number two.

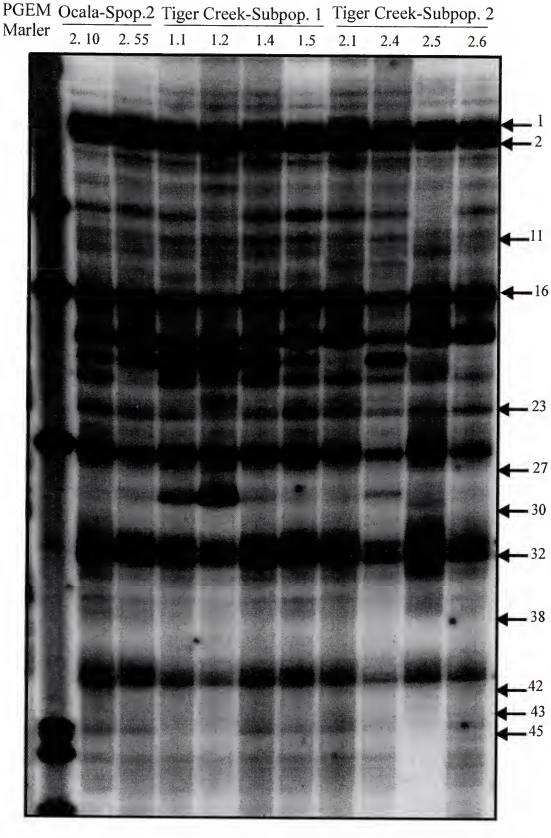

PGEM Marler
Ocala-Spop.2
2. 10
2. 55
Tiger Creek-Subpop. 1
1.1
1.2
1.4
1.5
Tiger Creek-Subpop. 2
2.1
2.4
2.5
2.6
1
2
11
16
23
27
30
32
38
42
43
45

Figure 4-26. Autoradiograph of AFLP from *Bonamia grandiflora* generated using the primer combination Mse I-CGA/EcoRI-CAC. One of the samples was collected in the Ocala National Forest, subpopulation three, and the rest were collected in the Sun Ray Preserve.

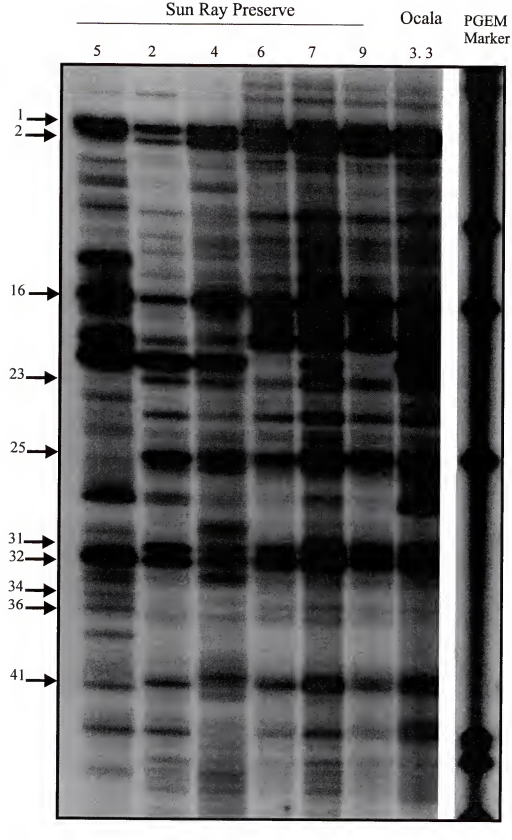

Sun Ray Preserve
Ocala
PGEM Marker
5
2
4
6
7
9
3. 3
1
2
16
23
25
31
32
34
36
41

Figure 4-27. Autoradiograph of AFLP from *Bonamia grandiflora* generated using the primer combination Mse I-CGA/EcoRI-CAC. The samples were collected in the Fenton Road and Hull Road populations.

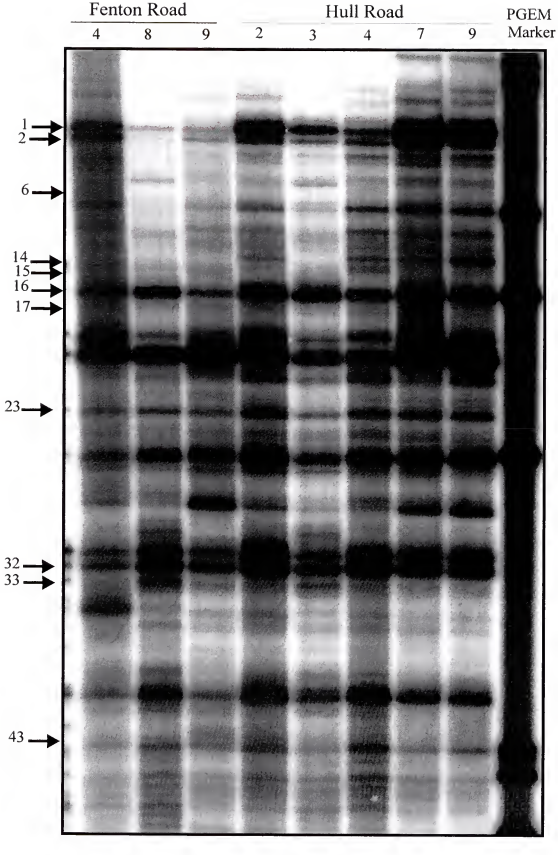

Fenton Road
Hull Road
PGEM Marker
4
8
9
2
3
4
7
9
1
2
6
14
15
16
17
23
32
33
43

Figure 4-28. Autoradiograph of AFLP from *Bonamia grandiflora* generated using the primer combination *Mse*I-CGA/*Eco*RI-CAC. One of the samples, *Stylisma* is the genus used as the outgroup, the rest belong to the Saddleblanket Preserve, subpopulation one.

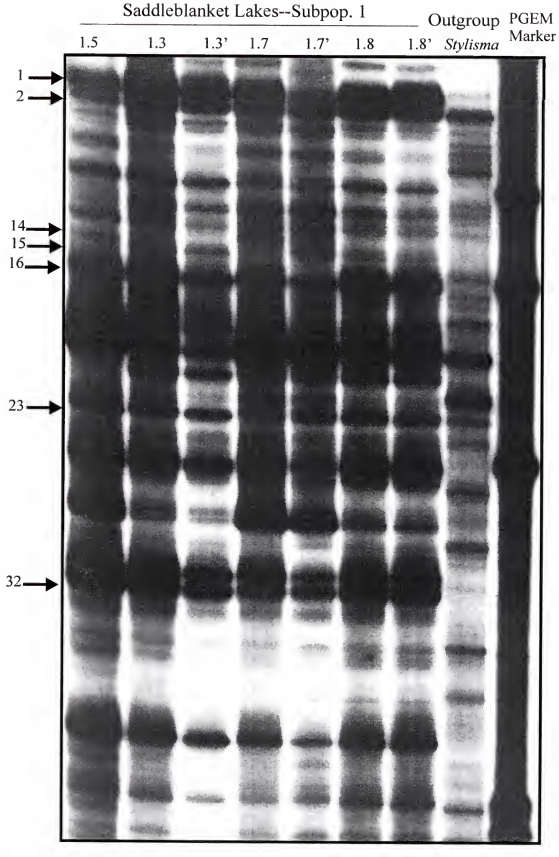
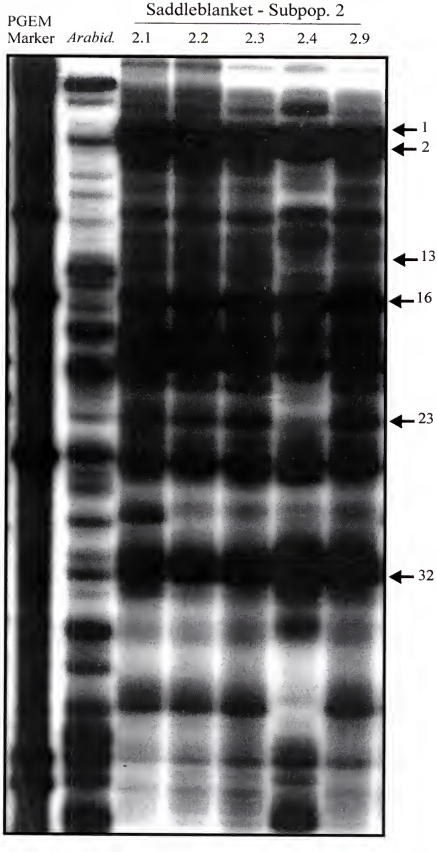

Saddleblanket Lakes--Subpop. 1
Outgroup
PGEM Marker
1.5
1.3
1.3'
1.7
1.7'
1.8
1.8'
Stylisma
1
2
14
15
16
23
32

Figure 4-29. Autoradiograph of AFLP from *Bonamia grandiflora* generated using the primer combination Mse I-CGA/EcoRI-CAC. One of the samples, *Arabidopsis*, is included as a comparison; the rest belong to the Saddleblanket Preserve, subpopulation two.


Saddleblanket - Subpop. 2
PGEM Marker
Arabid.
2.1
2.2
2.3
2.4
2.9
1
2
13
16
23
32

samples were consistent; even if the overall intensity of the bands varied, the banding profiles were identical (Fig. 4-23 and 4-28).

Of the forty-two individuals of *B. grandiflora,* thirty-eight presented unique fingerprints. Much of the variation is attributable to differences in the frequency of the markers between the subpopulations or populations. The homogeneity tests of forty-six AFLP loci revealed that 39.13% of them had different frequencies in different populations (Fig. 4-30).

Some of the bands were totally absent in some populations. For example, Ocala and Tiger Creek individuals lacked bands 27, 38, and 42 (Fig. 4-23, 4-24, 4-25, 4-26 and 4-30); Tiger Creek and Hull lacked band 43 (Fig. 4-25, 4-27, and 4-30), Hull and Fenton lacked band 17 (Fig. 4-27 and 4-30), Ocala also lacked band 30 (Fig. 4-23, 4-24, 4-25, 4-26 and 4-30), Tiger Creek lacked 45 (Fig. 4-25 and 4-30), and Hull lacked band 6 (Fig. 4-27 and 4-30). There were some bands found in only one individual and therefore one population; their frequency was less than 0.025. An individual in Sun Ray, SR5, had five of the eight rare alleles (absence of bands 25, 31, and presence of 34, 36, and 41) (Fig. 4-26); three other populations had one rare allele each and they were: Ocala, individual OII1 (absence of band 24, Fig. 4-24), Fenton Road, individual F4 (absence of band 33, Fig. 4-27), and Saddleblanket, individual SB2.4 (band 13, Fig. 4-29).

Molecular diversity is variable between *B. grandiflora* populations. Five of the loci were monomorphic across all the samples (bands 1, 2, 16, 23, and 32, Fig. 4-23 to 4-29 and 4-30). Tiger Creek and Hull Road had the lowest percentage of polymorphic bands, 26.09. When individual subpopulations are considered, the percentages of polymorphic bands are even lower, for example for OC1 and TC1 6.52% (Table 4-2).

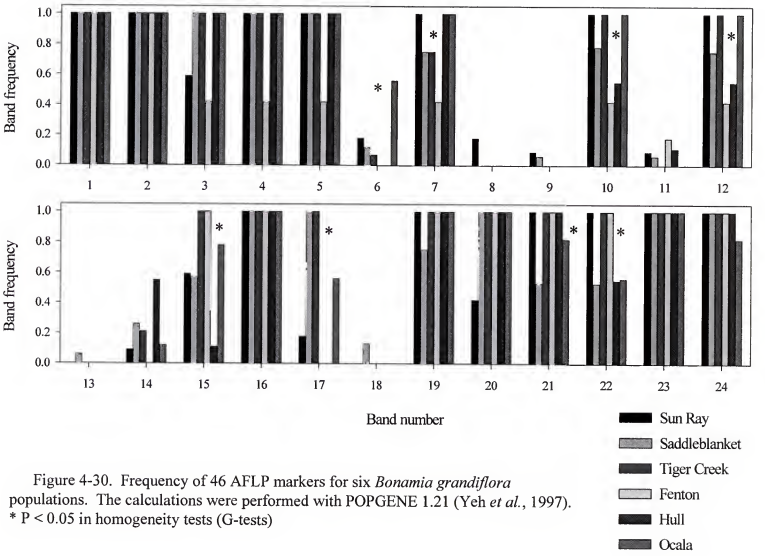


Figure 4-30. Frequency of 46 AFLP markers for six *Bonamia grandiflora* populations. The calculations were performed with POPGENE 1.21 (Yeh *et al.*, 1997). * $P < 0.05$ in homogeneity tests (G-tests)

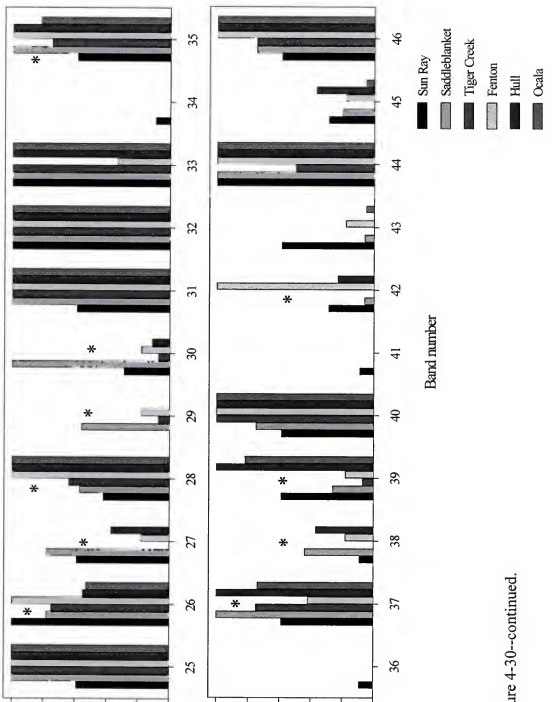


Figure 4-30--continued.

On the other hand, Sun Ray had the highest diversity, 56.52% and Saddleblanket followed, 52.17% (Table 4-2). There was a relatively weak but significant relationship between the percentage of polymorphic bands and the sample sizes ($r^2 = 0.45$, $p < 0.05$).

Table 4-2. Percentage of polymorphic AFLP bands in subpopulations and populations of *Bonamia grandiflora*.

Population	% polymorphic bands	Subpopulations	% polymorphic bands	N*
Sun Ray	56.52			6
Saddleblanket	52.17	SB1	21.74	4
		SB2	36.96	5
Tiger Creek	26.09	TC1	6.52	4
		TC2	23.91	4
Fenton Road	34.78			3
Hull Road	26.09			5
Ocala National Forest	28.26	OC1	6.52	3
		OC2	19.57	4
		OC2	10.87	4

* N = Number of individuals.

B. grandiflora shared twelve bands of identical length with *Stylisma*, the genus used as an outgroup (Fig. 4-28); two of the shared bands were the monomorphic markers 2 and 23.

As it was noted by Vos *et al.* (1995), the control lanes (no DNA template) still produced amplification products. None of these products had the same molecular weight as the five monomorphic markers (Fig. 4-23).

Sun Ray had the highest genetic diversity as expressed by expected heterozygosity 0.20 (Table 4-2). Saddleblanket was the second most diverse population, with a value of 0.17 (Table 4-3). At the other end of the spectrum was Tiger Creek with 0.08; Hull

Road also had low diversity, 0.10 (Table 4-3). Ocala had relatively low expected heterozygosity, 0.09 (Table 4-3). Fenton Road presented an intermediate value 0.13, (Table 4-3).

Table 4-3. Genetic diversity and estimated gene flow in 6 populations (10 subpopulations) of *Bonamia grandiflora*. All the calculations were done with POPGENE 1.21 (Yeh *et al.*, 1997); Hardy-Weinberg equilibrium was assumed.

Population		N*	na*	ne*	H_t*	H_s*	G_{st}*	Nm*
Sun Ray	Mean	6	1.55	1.36	0.20	0.20	0.00	2000
	St.Dev.		0.50	0.41	0.05	0.05		
Saddle-	Mean	9	1.51	1.28	0.17	0.11	0.34	0.99
blanket	St.Dev.		0.51	0.36	0.04	0.02		
Tiger	Mean	8	1.26	1.13	0.08	0.06	0.25	1.49
Creek	St.Dev.		0.44	0.26	0.02	0.01		
Fenton	Mean	3	1.34	1.24	0.13	0.13	0.00	2000
Road	St. Dev		0.48	0.37	0.04	0.04		
Hull	Mean	5	1.26	1.19	0.10	0.10	0.00	2000
Road	St. Dev		0.44	0.36	0.04	0.04		
Ocala	Mean	11	1.28	1.15	0.09	0.05	0.44	0.65
Nat. For.	St.Dev.		0.45	0.30	0.03	0.01		

* N = Number of individuals.
* na = Average observed number of alleles.
* ne = Average effective number of alleles (Kimura and Crow, 1964).
* H_t = Expected heterozygosity of an individual in an equivalent random mating total population.
* H_s = Expected heterozygosity of an individual in an equivalent random mating subpopulation (its value is identical to Ht if the population lacks subpopulations in the sampling scheme).
* G_{st} = Coefficient of gene differentiation; D_{st}/ H_t, proportion of the genetic diversity between populations relative to the total genetic diversity (it is zero if there are no subpopulations in the population).
* Nm = estimate of gene flow from G_{st}, $Nm = 0.5(1 - G_{st})/G_{st}$ (McDermott and McDonald, 1993), in number of migrants per generation.

Expected heterozygosity varied among loci (Table 4-4). The average total heterozygosity was 0.19, the average within population diversity was 0.10, and the

average between population diversity was 0.09 (Table 4-4). The highest heterozygosity was found in loci 39 and 17 and the least in 1, 2, 16, 23, and 32, the monomorphic loci (Table 4-4). While the average gene flow was 1.42 migrants per generation (Table 4-4), only eleven of the markers produced Nm values higher than one and only six bands, number 9, 11, 13, 34, 36, and 41, had values higher than four migrants/generation .

The average diversity within subpopulations ($1 - G_{st}$) was 0.59 and the average between subpopulation diversity (G_{st}) was 0.41. Loci 29, 17, and 30 showed the most difference between subpopulations, 0.77, 0.75, and 0.71, respectively (Table 4-4).

Table 4-4. Gene diversity statistics (Nei, 1987) for 10 *Bonamia grandiflora* subpopulations for 46 loci detected with AFLP. All the calculations were done with POPGENE (Yeh *et al.*, 1997); Hardy-Weinberg equilibrium was assumed.

Locus	N	H_t *	H_s *	D_{st} *	G_{st} *	Nm *
1	42	0.00	0.00	0.00	***	***
2	42	0.00	0.00	0.00	***	***
3	42	0.18	0.10	0.08	0.45	0.60
4	42	0.11	0.05	0.06	0.55	0.41
5	42	0.11	0.05	0.06	0.55	0.41
6	42	0.36	0.19	0.17	0.48	0.54
7	42	0.26	0.15	0.11	0.43	0.67
8	42	0.04	0.03	0.01	0.17	2.47
9	42	0.04	0.03	0.01	0.08	5.79
10	42	0.26	0.15	0.11	0.43	0.67
11	42	0.09	0.08	0.01	0.09	5.18
12	42	0.25	0.15	0.10	0.41	0.71
13	42	0.02	0.02	0.00	0.10	4.71
14	42	0.32	0.25	0.07	0.22	1.76
15	42	0.41	0.13	0.28	0.68	0.24
16	42	0.00	0.00	0.00	***	***
17	42	0.48	0.12	0.36	0.75	0.17
18	42	0.04	0.03	0.01	0.21	1.91
19	42	0.09	0.05	0.04	0.42	0.69
20	42	0.11	0.05	0.06	0.55	0.41
21	42	0.25	0.15	0.10	0.40	0.76
22	42	0.38	0.24	0.14	0.38	0.83
23	42	0.00	0.00	0.00	***	***
24	42	0.10	0.05	0.05	0.47	0.56
25	42	0.08	0.05	0.03	0.38	0.81
26	42	0.41	0.22	0.19	0.46	0.58

Table 4-4—continued.

Locus	N	H_t *	H_S *	D_{st} *	G_{st} *	Nm *
27	42	0.39	0.17	0.22	0.55	0.41
28	42	0.33	0.13	0.20	0.62	0.31
29	42	0.23	0.05	0.18	0.77	0.15
30	42	0.40	0.11	0.29	0.71	0.20
31	42	0.08	0.05	0.03	0.38	0.81
32	42	0.00	0.00	0.00	***	***
33	42	0.11	0.05	0.06	0.55	0.41
34	42	0.02	0.02	0.00	0.08	5.82
35	42	0.24	0.15	0.09	0.39	0.79
36	42	0.02	0.02	0.00	0.08	5.82
37	42	0.34	0.19	0.15	0.45	0.61
38	42	0.27	0.09	0.18	0.66	0.25
39	42	0.50	0.23	0.27	0.54	0.42
40	42	0.16	0.10	0.06	0.38	0.83
41	42	0.02	0.02	0.00	0.08	5.82
42	42	0.28	0.10	0.18	0.64	0.28
43	42	0.19	0.12	0.07	0.33	1.00
44	42	0.18	0.10	0.08	0.44	0.63
45	42	0.24	0.19	0.05	0.19	2.07
46	42	0.23	0.15	0.08	0.37	0.85
Mean	***	**0.19**	**0.10**	**0.09**	**0.41**	**1.42**
St. Dev.	***	**0.15**	**0.07**	**0.09**	**0.20**	**1.79**

* N = Number of individuals.

* H_t = Expected heterozygosity of an individual in an equivalent random mating total population.

* H_s = Expected heterozygosity of an individual in an equivalent random mating subpopulation.

* D_{st} = Proportion of the genetic diversity between populations (H_t - H_s).

* G_{st} = Coefficient of gene differentiation; D_{st}/ H_t, proportion of the genetic diversity between populations relative to the total genetic diversity.

* Nm = Estimate of gene flow from G_{st}, $Nm = 0.5(1 - G_{st})/G_{st}$ (McDermott and McDonald, 1993), in number of migrants per generation.

The nested analysis of molecular variance (Excoffier, 1995) showed that almost 70% of the genetic variation in this species is found among individuals within subpopulations (Table 4-5). Less than 22% of the variation is attributable to population differences (Table 4-5). Additionally, there was little (approximately 8%) variation between subpopulations within the main populations or regions (Table 4-5).

Table 4-5. Phenetic analysis of molecular variance, AMOVA, (Excoffier, 1995), for 42 individuals of *Bonamia grandiflora* using 46 AFLP markers. The design is nested as the individuals belong to 6 populations and 10 subpopulations.

Source	df	SS	MS	Variance component	% Total	P-value*
Between populations	5	54.54	10.91	0.936	21.85	<0.001
Within populations (between subpopulations)	4	17.54	4.39	0.355	8.28	<0.001
Within subpopulations	32	95.75	2.99	2.992	69.87	<0.001
Total	41	167.83				

* probability of obtaining a more extreme component estimate by chance.

The consensus tree constructed by PHYLIP from the best one hundred trees built by PENNY showed that individuals that had very close relationships i.e., identical phenotypes or one locus was different, clustered together (Fig. 4-31). H2, H7, H9, OC 1.1, and OC 1.2 clustered together, and so did SR2, SR6, SR7, SR9, and OC 3.3, and SB2.1, SB2.2, and SB2.3 (Fig. 4-31). However, less related individuals within populations tended to cluster in different branches of the tree.

The minimum spanning network (MSN) produced by MINSPNET produced a large cluster with individuals from each of the six populations intermingling to some extent (Fig. 4-32). Tiger Creek and Ocala occupy the center of the network and Sun Ray, Saddleblanket, Fenton and Hull, the periphery. In some of the populations, one or two individuals were separated from the others, such as in Fenton, Saddleblanket, Hull, Sun Ray (Fig. 4-32). Tiger Creek has five connections to Saddleblanket and one to Ocala and Fenton. Ocala has two connections to Hull and Sun Ray, and one to Tiger Creek. Saddleblanket has five connections to Tiger Creek, one to Sun Ray, and one to

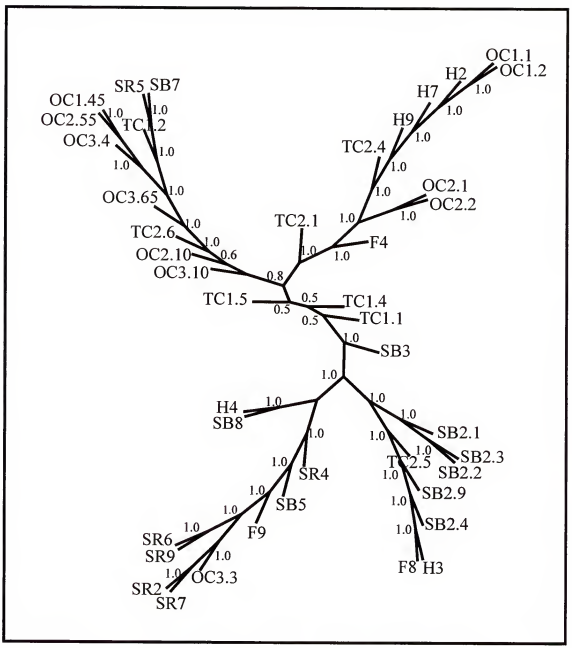
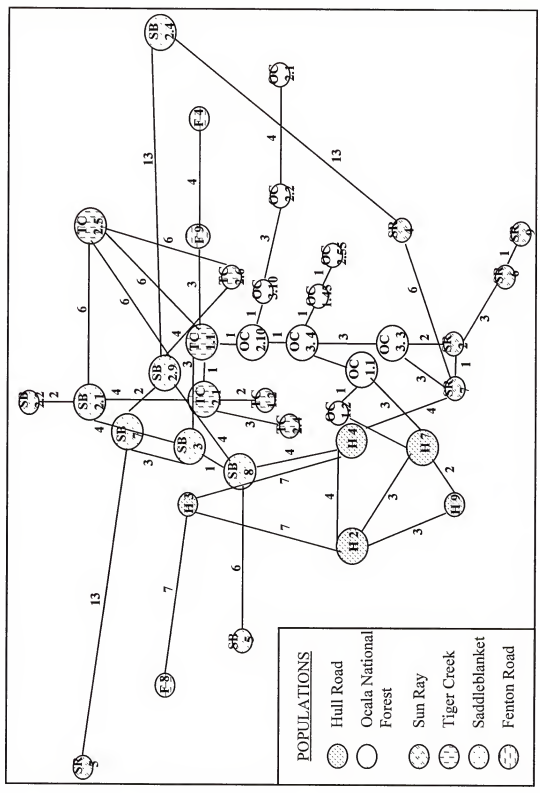


Figure 4.31. Consensus tree of 42 individuals of *Bonamia grandiflora* sampled in six populations in the Florida scrub. The consensus tree was built out of 100 trees constructed by PENNY, most parsimonious trees by branch and bound method (Felsenstein, 1993). OC1, OC2, OC3 = Ocala National Forest subpopulations; H = Hull Road population; TC1, TC2 = Tiger Creek Preserve subpopulations; SR = Sun Ray Preserve; SB1, SB2 = Saddleblanket Lakes Preserve subpopulations; F = Fenton Road population.

Figure 4-32. Minimum Spanning Network for 38 distinct individuals of *Bonamia grandiflora* based on AFLP fingerprinting. The numbers above the connections between the individuals are the numbers of differences between them.


POPULATIONS
Hull Road
Ocala National Forest
Sun Ray
Tiger Creek
Saddleblanket
Fenton Road

Hull. Sun Ray has two ties to Ocala and one to Saddleblanket. Fenton individual number eight, Saddleblanket numbers five and four in the second subpopulation, and especially Sun Ray number five are isolated from the rest of the individuals (Fig. 4-32).

The UPGMA cluster analysis of the AFLP markers (Fig. 4-33) shows little agreement with the UPGMA tree of geographic distances between populations (Fig. 4-34). The geographic and genetic distances were not correlated ($r^2 = 0.01$, $p = 0.46$). Hull clustered with OC1 and OC3, TC1 and TC2 clustered with OC2, and SB1 and SB2 did not appear closely related in the genetic tree. Geographically all the subpopulations were closer to each other than to other populations.

Discussion

Enzyme electrophoresis and RAPDs revealed little variation in *Bonamia grandiflora* populations. Although these results are unusual, they are not unprecedented. Detection of genetic variation is limited when using enzymes and lack of diversity has been found in other rare species such as *Pedicularis furbishiae* (Waller *et al.*, 1987), *Howelia aquatilis* (Lesica *et al.*, 1988), and *Lactoris fernandeziana* (Brauner *et al.*, 1992). RAPDs have also failed to detect diversity in cacao cultivars (Perry *et al.*, 1998), in *Limonium cavanillesii* (Palacios and González-Candelas, 1997), and within populations of *Saxifraga cernua* (Bauert *et al.*, 1998) . Lack of variation in RAPD markers in this study may be due to the increased stringency in annealing temperatures used in this study (40 to 46° C) in an attempt to prevent spurious products and to increase the reproducibility of the amplification profiles. Other authors have confronted

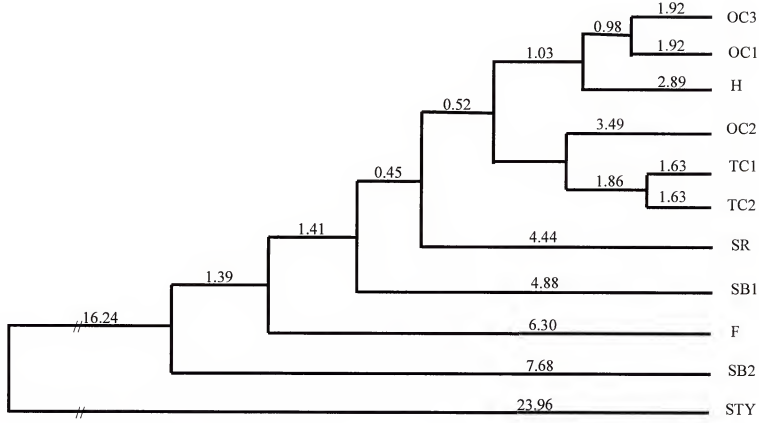


Figure 4-33. UPGMA cluster analysis of Nei's genetic distance (1978) for 10 populations of *Bonamia grandiflora* (42 individuals) and *Stylisma* as an outgroup; it is based on 46 AFLP markers. POPGENE 1.21 (modified from PHYLIP Version 3.5) (Yeh, *et al.,* 1997). OC1, OC2, OC3 = Ocala National Forest subpopulations; H = Hull Road population; TC1, TC2 = Tiger Creek Preserve subpopulations; SR = Sun Ray Preserve; SB1, SB2 = Saddleblanket Lakes Preserve subpopulations; F = Fenton Road population; Sty = *Stylisma* sp. (outgroup). The numbers above the branches show branch length.

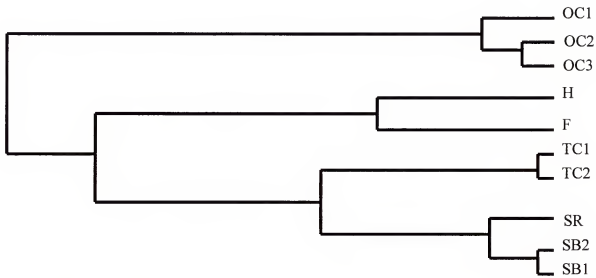


Figure 4-34. UPGMA cluster analysis of the geographic distances for 10 populations of *Bonamia grandiflora*. The distances are the approximated shortest distances between pairs of populations and were measured on maps. OC1, OC2, OC3 = Ocala National Forest subpopulations; H = Hull Road population; TC1, TC2 = Tiger Creek Preserve subpopulations; SR = Sun Ray Preserve; SB1, SB2 = Saddleblanket Lakes Preserve subpopulations; F = Fenton Road population.

a similar situation in another rare species, *Limonium cavanillesii* (Palacios and González-Candelas, 1997) after discarding non-reproducible bands they found no variation in RAPD markers. Similar to the situation found in *Limonium cavanillesii* (Palacios and González-Candelas, 1997 b), *L. dufourii* (Palacios and González-Candelas, 1997 a), and cacao (Perry *et al.*, 1998), *B. grandiflora* band reproducibility presented serious problems, even when strict standard conditions in the amplification were used.

In *B. grandiflora,* AFLP markers were more reliable than RAPDs and highly reproducible as shown by replicated samples. AFLPs also showed better discrimination among individuals than RAPDs and enzymes. Of the 42 individuals, 38 presented unique profiles. Identical profiles were found only within a particular subpopulation not between populations or regions. There some fixed differences between populations, that is, there were some population markers; Ocala, Tiger Creek and Hull lacked one band each and pairs of populations lacked several markers.

Calculations of expected heterozygosity (H_t and H_s), coefficient of gene differentiation (G_{st}) and gene flow (Nm) were done assuming Hardy-Weinberg equilibrium. When a F_{is} = 0.20 was assumed, H_t, H_s, G_{st} and Nm were not altered greatly; for example, for the Ocala subpopulations H_t decreased from 0.090 to 0.087; H_s remained at 0.51; G_{st} decreased from 0.437 to 0.408; and Nm increased from 0.644 to 0.7251. This suggests that even if the populations deviate from Hardy-Weinberg equilibrium, the estimates presented here are robust.

Overall, *B. grandiflora* had 37% polymorphic loci when it was analyzed with AFLP. This figure is very close to those calculated for near-isogenic lines of soybean (36%) (Maughan *et al.*, 1996), and for another rare plant, *Astragalus cremnophylax* var

cremnophylax (Travis *et al.*, 1996); both studies employed AFLPs. This indicates low genetic diversity in *B. grandiflora* and supports the findings with isozymes and RAPDs, which suggested very little genetic variation. There are a few other studies done in natural populations using AFLP markers; non-endangered species had much higher polymorphism averages, for example, *Cocos nucifera* was 61.4% polymorphic (Perera *et al.*, 1998), *Calycophyllum spruceanum*, a riverine Amazonian tree species had 91% polymorphic bands (Russell *et al.*, 1999), and a clonal species of *Rhododendron* exhibited 68% polymorphism (Escaravage *et al.,* 1998).

A group of scrub endemics, *Polygonella* spp., *Eryngium cuneifolium*, *Dicerandra frutescens*, *D. cornutissima*, *D. immaculata* and *D. christmanii* showed relatively high genetic diversity when enzyme electrophoresis was used, 21 to 54% polymorphic loci in *Polygonella* spp., 65% in *Dicerandra* spp., and 32% in *Eryngium cuneifolium*, (Lewis and Crawford, 1995; McDonald and Hamrick, 1996). In contrast, *Warea carteri,* another scrub endemic had reduced levels of genetic diversity; approximately 24% polymorphic loci for the species as a whole (Evans *et al.*, 1996). *Nolina brittoniana, Hypericum cumulicola, Liatris ohlingerae* had somewhat higher percentages of polymorphic loci than *W. carteri*, but still showed limited genetic variation (Menges *et al.*, 1996). Because isozymes, which have been shown to have less discriminating power among individuals than AFLP markers, were used in all these studies, it can be concluded that *B. grandiflora* probably presents as little genetic variability as *Warea carteri* and perhaps even less. Additionally, *B. grandiflora* is more widespread than most of the scrub endemics mentioned above.

In *B. grandiflora* populations where subpopulations were sampled, the smaller scale sampling always showed a lower percentage of polymorphic bands than the larger scale. This indicates that there are more monomorphic bands within each subpopulation than if the entire population is considered and shows that there is some local genetic stratification (Table 4-2). The same genetic structure can be seen when comparing expected heterozygosity for populations and subpopulations (Table 4-3).

In the AFLP analysis approximately a two-fold difference was observed between the populations with the most and the least genetic diversity (Sun Ray vs. Tiger Creek and Hull, Tables 4-2 and 4-3). The percentage of polymorphic loci and the expected heterozygosity (Tables 4-2 and 4-3, respectively) show similar trends. In both cases Sun Ray was the most diverse population, followed by Saddleblanket, and Fenton; Tiger Creek was the least diverse. Hull showed higher diversity than Ocala as expressed by expected heterozygosity, but the reverse was true when considering percentage of polymorphic bands. Sample size and size of the populations did not appear correlated to either expression of diversity.

Although the SR and SB are not large in number of individuals or geographically widespread they both have the highest genetic diversity of the populations samples; both populations have high expected heterozygosity and high percentage of polymorphic loci (Table 4-2 and 4-3). Gst (Table 4-3) also showed great subpopulation differentiation in SB.

Population genetic structure was also explored by using AMOVA and Nei's gene diversity index calculated by locus. AMOVA showed a within subpopulation variation

of almost 70% and Nei's index were almost 60% (± 20%). Although the two measures do not agree very closely, it is clear that most of the diversity in *B. grandiflora* is found among individuals within subpopulations. Similar results have been shown in another rare species from Australia, *Grevillea barklyana*, in which 80% of the variation was found among individuals (Hogbin *et al.*, 1998). In *Lantana,* 74% of the diversity was found within populations (Scott *et al.*, 1997), in *Calycophyllum spruceanum* (Russell *et al.*, 1999) 91% of its genetic diversity was allocated within populations, in wild *Hordeum* (barley), 57% of the variation was within populations (Dawson *et al.*, 1993). Conversely, in *Astragalus cremnophylax* most of the variation was found between populations (Travis *et al.*, 1996) or in *Moringa oleifera* in which 60% of the diversity was between populations (Muluvi *et al.*, 1999). It is unknown why the allocation of genetic diversity is different in these species. The AMOVA analysis shows that most of the residual the variation is found between populations (approximately 22%) and little variation is shown between subpopulations (8%).

The average gene flow in *B. grandiflora* was 1.42 migrants/generation (Table 4-4). The majority of the bands, however, presented values of less than one while six of the markers indicated gene flow above four migrants/generation. Of those six bands, three are rare alleles only present in individual SR 5, one is only present in SB 2.4, and the other two are present in SB 2.4, SR 4, H3, and F8. Excluding the few alleles that have large Nm values, the average gene flow is 0.72 migrants/generation. It is generally believed that at gene flow values below two (Hartl 1988) or one migrant/generation (Slatkin 1987), the effects of genetic drift become important. At present, *B. grandiflora*

appears relatively homogeneous but given the reduced values of gene flow it can be expected that populations will differentiate further in the future.

A comparison of the genetic (Fig. 4-33) and geographic UPGMA (Fig. 4-34) dendrograms shows that the agreement between the two is rather poor ($r^2 = 0.01$, $p = 0.46$). For example, OC2 is genetically more related to TC1 and TC2 than it is to OC1 and OC3; the last two cluster with H instead. SR appears more closely related to the above mentioned populations than to SB1 and SB2. SR is approximately 7 km from SB but 23 km from TC, the closest of all other populations. In the genetic dendrogram, SB1 and SB2 are not closely related. Poor correspondence between geographic and genetic distance has been found in studies of *Astragalus* (Travis *et al.*, 1996), *Lantana* (Scott *et al.*, 1997) and *Limonium* (Palacios and González-Candelas, 1997 b), root knot nematodes (Semblat *et al.*, 1998), and *Calycophyllum spruceanum* (Russell *et al.*, 1999). This indicates that genetic variation is not stratified in a geographic manner; again, because most of the variability is found within populations, the reduced differences between populations are insufficient to define regions clearly. The lack of correlation between geographic and genetic distances in *Calycophyllum spruceanum* was also attributed to most of its genetic diversity being found within populations (Russell *et al.*, 1999).

Phylogenetic trees are often used to reflect relationships among individuals of a species, for example, in *Moringa oleifera* (Muluvi *et al.*, 1999). However, in the case of *B. grandiflora*, the PENNY phylogenetic tree does not appear to adequately reflect intraspecific relations. The consensus tree (Fig. 4-31) agrees poorly with the MSN (Fig. 4-32). The phylogenetic tree and MSN agreed on joining some individuals that have a

difference in one locus such as OC 1.45 and OC 2.55, OC 1.1 and OC 1.2 but PENNY failed to cluster some individuals with only one difference between them. For example, SB8 and SB3 have only one difference but in the tree SB8 is joined to H4, from which it is four differences apart. The clustering of F8, H3, H2, and H4 is poor in the consensus tree. Because in tree construction only bifurcating branches are normally used, phylogenetic trees may not be appropriate to display population genetic diversity when most of the variation is found within populations. In this case, MSNs can show intraspecific diversity better than trees because MSNs are capable of reflecting relationships among several individuals simultaneously and no choice is forced on its groupings. The inadequacy of phylogenetic trees to reflect intraspecific relationships when using RAPD markers has already been noted in *Limonium* (Palacios and González-Candelas, 1997 a).

There are probably hundreds of surveys of genetic diversity in natural populations. The majority of them have used enzyme electrophoresis because, for many years, it was the only technique available. However, this is likely to change in the future since several techniques to assess genetic diversity in populations have been developed. Regardless of the method used it is necessary to be able to compare the results in order to understand better the distribution of genetic diversity in suites of species and perhaps gain a better understanding of population dynamics and biogeographic processes. To enable these comparisons it is indispensable to know the power of discrimination among genotypes that the techniques to assess genetic diversity have and their potential biases.

In this study, only one of the five enzymes (GPI) revealed a few individuals to be different from the rest in the Ocala National Forest. RAPDs also showed very little

diversity with the seven primers used in Ocala National Forest. AFLPs, however, produced over 28% of polymorphic markers within the Ocala National Forest and all but two of the individuals had unique fingerprints, even when the sampl sizes used were much smaller in the AFLP analysis than with RAPDs or enzymes.

Comparing results from samples of different Florida populations, thirteen RAPD primers revealed only a few rare alleles but AFLPs showed an average of 37% polymorphism. Clearly, results from genetic surveys obtained by different techniques cannot be directly compared. Other studies produced similar conclusions, for example, in *Calamagrostis imperata sp. insperata*, a rare clonal grass, only 6% of the allozymes were polymorphic but 22.5% of the RAPD bands and 24% of ISSR markers were found to be polymorphic (Esselman et al., 1999) and in *Moringa oleifera* (Muluvi *et al.*, 1999) the genetic diversity found with AFLP was three times as much as that uncovered by RAPD markers.

The power of the molecular technique used has direct impact on the recommendations made for species management (Esselman *et al.*, 1999). In *B. grandiflora* recommendations based on enzyme electrophoresis and RAPD analyses would have been that relocation or recolonization within the Ocala National Forest and even between Florida populations (based on RAPDs) would not alter the genetic structure of the populations, except for a few rare alleles. Recommendations based on the AFLP analysis are radically different since genetic structure has been detected in *B. grandiflora* subpopulations and populations. When choosing a seed source to recolonize areas or to establish new populations the closest population should be used as all populations were shown to have different allele frequencies and several private alleles.

Hamrick and Godt (1990) and Hamrick *et al.* (1991) provided correlations between species traits and allozyme diversity in plants. Their work, based on studies of 449 plant species, provides estimates of average polymorphism (P), expected heterozygosity, and the proportion of total diversity found among populations (G_{st}). *B. grandiflora* had 37% polymorphic loci and expected heterozygosity for subpopulations, H_s, of 0.10 when AFLP markers were used. Hamrick and colleagues, using allozyme data, calculated averages of 40% polymorphic loci for endemic species, 14.4% for species with mixed breeding systems and animal pollinated, 39.6% for herbaceous perennials and 45.7% for gravity dispersed (Hamrick and Godt, 1990); the genetic diversity index, He, was 0.096 for endemics (Hamrick *et al.*, 1991). While G_{st} for *B. grandiflora* (average for 46 loci) was 0.41, G_{st} was 0.21 for animal pollinated species (Hamrick *et al.*, 1991). *B. grandiflora*'s percentage of polymorphic loci is below those of endemic species, herbaceous perennials and gravity dispersed plants. However, several of the average estimates by Hamrick and Godt (1990) are lower than those of *B. grandiflora*. The same pattern was noted in AFLP marker data for *Astragalus cremnophylax* by Travis *et al.* (1996); in this rare and critically endangered species P = 0.38 and H_t = 0.13.

Again, allozyme data and data obtained from AFLP markers are not directly comparable. Average number of alleles, for example, is a valuable measure in enzyme electrophoresis studies but meaningless in AFLPs because it is assumed that each gene has two alleles, the presence or absence of a band. It is also apparent that AFLP markers produce significantly higher percent polymorphism and heterozygosity than enzymes but the exact magnitude of the difference is unknown because of the lack of comparative studies. In this work, Ocala populations were 20% polymorphic with enzymes and

28.26% with AFLP data. If a linear relationship existed between enzyme and AFLP data, 26% polymorphism could be expected for the species as a whole. However, the exact relation between these two types of markers is unknown and the sample sizes used in the two stages of this study are too dissimilar to draw any conclusions (56 individuals were sampled in the Ocala National Forest for enzyme electrophoresis but only 11 were sampled for AFLP analysis).

The Ocala populations of *B. grandiflora* are the largest but their genetic diversity is relatively low. At the southern end of *B. grandiflora*'s distribution, the Sun Ray and the Saddleblanket Preserves have populations with the greatest genetic diversity, but the present sizes of those populations are relatively small. The Tiger Creek Preserve, and Hull and Fenton, which are approximately central to the present distribution have intermediate to low diversity. This distribution of genetic diversity is peculiar and it may be due to historical factors.

It has been proposed that scrublike habitats expanded during glaciations and contracted during interglacial periods (Ch. 1; Delcourt and Delcourt, 1981) and that some populations of scrub species were probably able to survive the warmer periods in localized refugia (Lewis and Crawford, 1995). Both, the numerous endemic species found in the Florida scrub and the high genetic diversity found in Florida species of *Polygonella* may be explained by the existence of these refugia (Lewis and Crawford, 1995).

It is unclear where these refugia were located because most palynological records in Florida have a very large gap between 35 000 and 8 100 B.P., probably due to a low water table during glacial periods (Watts, 1971; Watts, 1969). Pollen records from Mud

lake in Marion Co. (within the Ocala National Forest scrub) and Scott lake in Polk Co. (close to the Lake Wales Ridge) show this discontinuity (Watts, 1971; Watts, 1969). However, the profile found in a lake in Highlands Co., Lake Annie (on the Lake Wales Ridge) has a continuous deposit of pollen from the Pleistocene to the present (Watts, 1975). The palynological profile of lake Annie shows the persistence of scrub genera such as *Ceratiola*, *Selaginella*, *Polygonella*, and *Serenoa* from 37 000 BP to the present (Watts, 1975). It is possible that refugia were only found at the southernmost end of present scrub and *B. grandiflora* and other scrub species persisted in those small areas of the Lake Wales Ridge during glaciations. During interglacial periods the populations expanded north again, but not all the alleles present in *B. grandiflora*'s southern populations managed to reach northern scrubs such as Ocala. This hypothesis can be tested by investigating the genetic structure of other scrub endemics that are not limited to the Lake Wales Ridge such as *Polygala lewtonii*, *Asclepias curtissi*, *Carya floridana*, *Ilex opaca* var. *arenicola*, or *Persea humilis* among others.

Other scrub plant species must also have suffered the effects of glaciations, but the genetic diversity of some of those species is relatively high. Therefore, the bottleneck during glaciation seems insufficient to explain *B. grandiflora*'s very low genetic diversity.

There may be other reasons to account for *B. grandiflora*'s low genetic diversity. Many *Bonamia* species are limited to sandy, xeric areas and several are also rare. This suggests that the genus as a whole may have limited capacity to adapt to novel environments, and could have limited evolutionary potential.

In several species it has been shown that the centers of distribution, or central populations, have higher marker diversity than peripheral populations, for example, in

wild tomatoes (Villand *et al.,* 1998); in *Acacia melanoxylon* (Playford *et al.,* 1993), although in this case there may also be a latitudinal effect; in house mice (Boursot, *et al.,* 1996); and in the least shrew (Hafner and Schuster, 1996). Because the genus *Bonamia* migrated from South and Central America into Florida, probably progressing around the Gulf of Mexico, it may have reached the peninsula with an already depauperate gene pool.

Additionally, it is also possible that in the harsh environmental conditions of the scrub stabilizing selection may act on *B. grandiflora* by removing new genetic variants. Although the area occupied by scrub has varied greatly, the environmental conditions experienced by *B. grandiflora* may have remained relatively constant for thousands of years.

A similar scenario has been suggested by Ricklefs and Latham (1992). These authors analyzed disjunct genera in eastern Asia and eastern North America, both woody plants and perennial herbs and they noted high parallelism in the geographic range of the herbs but not in woody plants (Ricklefs and Latham, 1992). These authors proposed the following explanation: the perennial herbs exhibiting parallelism are forest inhabitants, with very particular ecological requirements. As environmental conditions changed, these herbs either persisted wherever they could fulfill all their requirements or they were extirpated. On the other hand, woody taxa in the same areas were, supposedly, more generalized and able to respond to directional selection (Ricklefs and Latham, 1992). Long-term genetic stasis has been also been demonstrated in gastropod and bivalves of the Cretaceous period (Jablonsky, 1987).

Howellia aquatilis is another endangered species with a relatively large and fragmented geographic range that has shown no apparent genetic polymorphism even when eighteen isozymic loci were tested in four populations and sample sizes were large (Lesica, 1992). *Howellia* is only found in ponds or sloughs that lack an outlet and are probably accumulating sediments (Lesica *et al.*, 1988). These ponds dry or nearly dry up late in the growing season, and they are rich in organic matter, and at least partially surrounded by deciduous trees. Obviously, these factors are related but the authors did not know which one or ones were fundamental (Lesica *et al.*, 1988).

There is a common feature between Ricklefs and Latham's perennial herbs, *B. grandiflora* and *Howellia aquatilis*, and it is the very specialized environmental conditions in which all these species live. There seems to be a relationship between rarity and specialized requirements. However, it would be impossible to know if genetic impoverishment is the cause of narrow ecological amplitude or its effect.

B. grandiflora showed weak genetic structure in its populations as most of the variation was found among individuals. The simplest explanation of this weak genetic stratification is that not enough time has elapsed since the populations became isolated.

Weak population differentiation in *B. grandiflora* may also be due to the existence gene flow. Very little gene flow is necessary to make populations homogeneous; an average of one individual per generation exchanged between populations will prevent neutral alleles at the same locus from being nearly fixed (Slatkin, 1987). Slatkin goes on to elaborate that this estimate is independent of population size, given that in larger populations the gene flow effect will be smaller, but then so will be the effect of genetic drift, consequently the two effects compensate each other.

Most of the loci analyzed indicate that there is not much gene flow among populations since, when atypical alleles are removed from the calculations, the average gene flow was 0.72 individuals per generation. However, both present gene flow and historical events, which influence the genetic structure of the populations, affect these estimates of gene flow.

One way to find out whether gene flow actually exists among populations is testing it directly; however, it would be very difficult and costly. Direct methods include the observation of dispersal of organisms or their seeds, pollen, sperm or eggs. This has been accomplished in a few organisms such as the checkerspot butterfly (Ehrlich, 1975). Observation of this species during many years indicated that there was no gene flow among subpopulations even at the smallest geographic scale. However, when a local population went extinct, the site was recolonized within a year (Ehrlich, 1975). This example indicates that gene flow can be higher than expected and is very difficult to assess.

Direct testing of gene flow in *B. grandiflora* would involve marking seeds or pollen. From the results of this study, it must be concluded that seed dispersal by animals is infrequent but possible but the vectors remain unknown. Dyes have been used to track small scale pollen dispersal in *Ipomopsis aggregata* (Campbell, 1991) and other species. It would not be possible, however, to use this technique to assess long distance pollen transport. A better option would be find out more about the home ranges and foraging behavior of *B. grandiflora*'s pollinators. Unfortunately, though larger than pollen and seeds, these pollinators are small, and tracking their movements would also be difficult. For example, Breden (1987) marked over 25 000 tadpoles and metamorphosing Fowler's

Toads to study their dispersal patterns. After five years of work, he recovered only 37 adults (Breden, 1987). Even with ponds concentrating these animals, marking and recapturing such high numbers of individuals is demanding in terms of time, labor, and funding. In the case of *B. grandiflora* no areas with high densities of pollinators are known.

An alternative method to directly assess gene flow could be applied if unique markers were present in a limited number of *B. grandiflora* individuals. An extensive genetic survey could track the transfer of those markers to new individuals. This method has been used successfully in wild radish among other species (Ellstrand *et al.*, 1989). This method to assess gene flow will probably be used more often in the future, although it is labor intensive and expensive.

On the other hand, several authors have concluded that genetic surveys are better estimators than dispersal studies, since the latter usually underestimate gene flow (Templeton *et al.*, 1990). Indirect testing of gene flow requires finding some genetic heterogeneity. Because some polymorphisms have been detected with AFLP markers in *B. grandiflora*, the calculation of gene flow was possible. Again, as it is possible that the genetic structure revealed by AFLP is a relict of the past so this information must be interpreted with caution.

Although most of the AFLP markers are dominant co-dominant loci have been verified in some studies where controlled crosses were performed. For example, in rice 10.6% of the markers were co-dominant (Maheswaran *et al.*, 1997). One of six AFLP markers tested in soybean was co-dominant (Maughan *et al.*, 1996), and from 6 to 12.6% in different mapping populations of barley (Waugh *et al.*, 1997). In those cases the co-

dominant bands were amplification products with very similar molecular weights. Bands with very similar molecular weight were observed in *B. grandiflora*: in the Sun Ray population bands 34 and 36 in individual number five had very similar length to bands 35 and 37 in the rest of the individuals and no individual had bands 34 and 35 or 36 and 37. Bands 14 and 15 present a similar case, the overwhelming majority of individuals present either one or the other, but four of the 42 individuals present both bands. Some of these bands could be co-dominant. If that were the case, the estimates of number of loci and heterozygosity would be incorrect. RAPD markers suffer the same liability and in studies such as this one, where no verification of the nature of the markers by crosses was done, some inaccuracy in the results is unavoidable.

It has been shown that different primer combinations produce different numbers of markers; for example, in *Astragalus cremnohylax* one primer pair produced 29 markers and another pair produced 58 (Travis *et al.*, 1996). In *Glycine max* and *Glycine soja* the number of markers produced per pair of primers ranged from 19 to 86 (Maughan *et al.*, 1996). On average AFLP primer pairs seem to produce about 45 markers; in *Lens* primer pairs produced on average 37 markers (Sharma *et al.*, 1996), in coconut 40.25 (Perera *et al.*, 1998) in cacao, the average number of markers generated by AFLP was 44 (Perry *et al.*, 1998), 47.3 in rice (Maheswaran *et al.*, 1997), in *Glycine max* and *Glycine soja* 50.6 (Maughan *et al.*, 1996). The results for *B. grandiflora* fit this generalization as 46 markers were produced by one primer pair.

It appears that different primer combinations lead to consistent conclusions about the patterns in genetic diversity. In a study of wild and cultivated *Lens* species (lentil), the authors published Nei's genetic identities based on four primer combinations

(Sharma *et al.*, 1996). Based on those, data it can be calculated that the average standard deviation between primers is low (0.045) and it may be unnecessary to fingerprint individuals with numerous primer combinations. However, depending on the application of the information sought or the homogeneity of the individuals under study, the use of more than one pair may be advisable. In the case of *Lens* somewhat better resolution in the phylogenetic tree was gained when markers from four primer combinations (148 markers) instead of one pair (23 markers) were used. Additionally, all the accessions were individually identified when using four pairs of primers but not when using only one pair (Sharma *et al.*, 1996).

A very important advantage of enzyme electrophoresis in population studies is that species can be compared when they are tested for the same enzymes, thus we can gain some understanding of the overall levels of genetic diversity in taxa with different life histories, geographic location, reproductive systems or other parameters of interest (Hamrick and Godt, 1990; Hamrick *et al.*, 1991). Another benefit of using enzyme electrophoresis is that the markers produced are co-dominant and Hardy-Weinberg equilibrium can be directly assessed in populations. Nevertheless, sometimes allozymes lack the resolving power to detect the underlying genetic variation and other markers must be used instead.

One of the attractive features of RAPDs was the potential to compare the genetic diversity of different species when the same primers were used to generate the fingerprints as it has been done with allozymes. RAPD markers have been used in phylogenetic analysis of species of *Glycine* (Williams *et al.*, 1990; Caetano-Anollés *et al.*, 1991), in *Brassica* species (Lannér *et al.*, 1996), in the *Lolium/Festuca* complex

(Stammers *et al.*, 1995) and in studies of the geographic distribution of species of *Lens* (Ferguson *et al.*, 1998) among others. However, because 1- the amplification conditions used are variable between the different studies (due to the need to optimize), 2- many primers are available, and 3- reproducibility of amplification profiles is low, it is practically impossible to compare any species that are not tested together.

AFLP markers can fill the need for reliable and highly discriminating markers in population studies. In the several studies cited above, the AFLP protocols employed were either identical or very similar to the original protocol (Vos *et al.*, 1995); this shows that if the template DNA is pure enough to be digested and amplified, the fingerprints obtained and population genetic statistics generated from them can be compared between species as it has been done in the past with allozymes. One obstacle to do this is that researchers use different adaptors and primer sets. Most of the authors have worked with *Eco*RI and *Mse*I adaptors and primers as Vos *et al.* did in the original work, but the selective bases were different in the different studies. A possible solution would be the use of commercial AFLP kits, already available, or the use of sequences that have appeared in the literature.

So far AFLP has been applied mainly to plant species, but the developers of this technique have also applied it to bacterial, yeast, and human DNA with success. It appears that in animal studies a different adapter-primer combination, based on *Eco*RI and *Taq*I renders better results (Ajmone Marsan *et al.*, 1997). Besides minor modifications AFLP seems readily applicable to a variety of taxa. Additionally, like RAPDs, AFLPs sample a large portion of the genome.

In the published literature AFLPs have been used as mainly dominant markers. Some commercial laboratories, however, claim to have developed techniques that make possible the distinction between homozygoud dominant and heterozygous individuals, supposedly by careful quantification of the signal produced by fluorescent tags. If the direct assessment of heterozygous individuals became possible, the value of AFLP would be greatly enhanced.

Summary

Genetic variation in *Bonamia grandiflora* is reduced and comparable to near-isogenic agronomic lines and very rare species. This low diversity could be explained by an impoverished initial gene pool or strong selection in a harsh environment or both.

The genetic diversity is allocated mainly among individuals and is consistent with some rare species (*Grevillea barklyana*) but in contrast to others (*Astragalus cremnophylax*). There is weak structuring of the genetic diversity and no single population covers its entire gamut. The weak stratification of genetic diversity in *B. grandiflora* may be due to lack of time since the fragmentation of the populations or due to gene flow. The first hypothesis seems more likely, since gene flow estimates gave an average of less than one migrant per generation.

Sun Ray and Saddleblanket, located in the southern end of the geographic distribution of *B. grandiflora*, have the greatest genetic richness. Ocala, despite having the largest populations has relatively low diversity. These facts could be explained if, during glacial periods, only southern populations persisted and served as sources for

recolonization during interglacial periods. This bottleneck must also have contributed to the overall low genetic diversity of *B. grandiflora*.

The minimum spanning network provides a very useful representation of the genetic diversity in *B. grandiflora* as it is capable of showing the intricate relationships among individual genotypes. On the other hand, phylogenetic trees did not show intraspecific relations appropriately in *B. grandiflora*; this is most likely due to most of the variation being allocated among individuals within populations.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

The reintroduction of threatened or endangered plant species into habitats from which they have been lost, as the result of human activity, is one aspect of ongoing efforts to redress the balance between maintaining biological diversity and human use of land. *Bonamia grandiflora* is one of many species with a narrow habitat range and distribution, being confined to the scrubland of the Central Ridge of Florida. Along with the other scrub species, this plant had been unaffected by human activity until very recently. It is only within the past 150 years that much of the scrubland has been used for growing citrus, for housing and for forestry. This has resulted in isolated areas of scrubland and isolated/fragmented populations of many scrubland species. Further changes in land use have made suitable habitats available again and so there is the opportunity for locally reintroducing lost species.

The most suitable source of seeds would be the seed bank already present in the reclaimed habitats. Such a source rarely survives the previous land use, as can be seen in many of the abandoned citrus groves in Florida. The next most suitable source would be from habitats as close as possible to the target site, but seed may well be more readily available from elsewhere. If there were little or no genetic variability in the species, the precise source of the seed would be of no consequence. On the other hand, if there were genetic variation in the species, the reintroduction is less likely to be successful as well as

creating esoteric problems of ecological genetics and population dynamics, let alone ethics.

The initial examination of plants in the seed orchard of the Ocala National Forest facility revealed some variation between individuals that had been derived from different sources. Because these plants were growing in a common garden arrangement there was *prime facie* evidence that there were genetic differences between these plants for the characters observed. The data and analysis in Chapter 2 documents that there is indeed some phenotypic variation in *B. grandiflora,* although it was not possible in this study to follow through with a breeding program. Thus, there was sufficient evidence to question the apparent lack of genetic diversity as revealed by the enzyme electrophoresis. The RAPD analysis would be expected to have improved the resolution, but again little variation in the DNA of the plants was revealed. At this stage, therefore, the conclusion was that the source of seed for the reintroduction program was not important, although it would be sensible to use seed from a source as close to the target habitat as possible.

Meanwhile the new technique of AFLP analysis had been introduced. The evidence presented in Chapter 4 shows that the AFLP analysis was both more reliable and had better resolving power than either enzyme electrophoresis or RAPD markers. Furthermore, of the forty-two individuals analyzed only two pairs shared the same profile. Thus, there is some genetic diversity in *B. grandiflora*. Consequently the advice to be given about the source of seed for the reintroduction program needs to be reassessed.

The phenotypic characterization of populations of B. *grandiflora* throughout its geographic range revealed differences between populations for some characters; those are

seed weight, seed dimensions, and the ratio of seed length to its width. Higher ratios of seed length to width appear to be associated with higher percentage germination and this relationship should be investigated further. The size of the plants was also different between Florida populations and these differences are probably attributable to historical, microenvironmental factors or random events.

Leaf pubescence and flower dimensions showed no differences between populations; this may indicate lack of genetic variation underlying these characters due either to general genotypic homogeneity in *B. grandiflora* populations or to strong stabilizing selection for these characters.

Total reproductive output was associated with the size of the plant and therefore it varied between populations, but other factors strongly influence this character as well; it is unknown if those factors are ecological or genetic or both. The reproductive output/stem length did not differ between populations. This may indicate that if inbreeding depression is influencing this aspect of the reproductive potential of *B. grandiflora,* it is affecting all the populations studied evenly. However, the variances in these data were large and results must be interpreted with caution.

Some inbreeding depression was verified in Ocala populations of B. *grandiflora*; it was mild and was found in the number of seed/fruit and percentage germination. However, based on estimates from other angiosperms (Husband and Schemske, 1996), five times more inbreeding depression can be expected at later stages of *B. grandiflora*'s life cycle.

B. grandiflora has a mixed mating system and it is capable of producing seeds apomictically. It does not have highly specialized pollinators so it is not susceptible to

the loss of particular insect species. However, significant seed production only occurs when pollinators are present, and some pollinators appear to favor other scrub species such as *Serenoa repens* when those species are blooming. If the number of pollinators available became limited in a particular season or population, *B. grandiflora* may experience decreased reproduction.

B. grandiflora fruits have relatively homogeneous weight regardless of the pollen source used but cross-pollinations produce larger number of seeds/fruits. Although those seeds are smaller than selfed seeds, they show the effects of outcrossing, because they exhibit higher germination than the latter.

Germination percentages were low overall and did not exceed 67% even when the seed coats were abraded and the seeds imbibed. Even in greenhouse conditions, it is difficult to maintain the correct moisture content in the germination medium during the ten to fifteen days that seedlings take to emerge; high humidity causes fungal damage in the seeds and low humidity prevents germination. Additionally, seeds did not germinate in Petri dishes on moist filter paper. All seeds that were used to initiate the tissue cultures (approximately 15 seeds) germinated when they were immersed in agar. This suggests that to germinate *B. grandiflora* seeds need to be buried in the germination medium. It also indicates that chemical dormancy in this species is unlikely.

In the field, most of the seeds are found within the upper centimeter of soil (Hartnett and Richardson, 1989). *B. grandiflora* seeds can be commonly found on the ground surface but they appear to germinate only when they are underground. In the hot and sunny environment of the scrub, seeds probably cannot stay moist unless they are protected by soil. Given that *B. grandiflora* grows in soils that are basically sand, with

little water holding capacity, the correct soil moisture conditions may occur rarely. This appears to be true even in more controlled conditions such as the greenhouse. The same observation has been made in sand dune species by Maun and Lapierre (1986).

Fire stimulates seed germination in *B. grandiflora* (Hartnett and Richardson, 1989). Fire may also enhance the survival of seedlings (D. Gordon personal communication) although the mechanism is unknown.

B. grandiflora forms seed banks and the number of seeds ranged from 1.9 to 30/m^2 in sites with different histories (Hartnett and Richardson, 1989). Because *B. grandiflora* is a perennial species, presumably the seed banks consist of seeds that come from plants that persisted through benign as well as adverse environmental conditions. Thus, seed banks could also maintain genetic diversity (Baker, 1989). It is unknown for how long *B. grandiflora* seeds remain viable but if they were very long lived, say a hundred years, and the mature plants themselves were old, perhaps 30 years, the genetic structure assessed in this study could reflect past conditions. If this were true, then the impact of recent habitat fragmentation due to development would not appear in this genetic survey and could be determined only in the future.

As mentioned above, management recommendations for *B. grandiflora* based solely on enzyme electrophoresis or RAPD markers would have been different from those based on AFLP markers. Thanks to the increased resolving power and high reliability of the AFLP markers, better decisions can be made about potential germoplasm relocation. While the three techniques indicate that low genetic variability is found in *B. grandiflora*, AFLP analysis detected the populations with the largest genetic diversity and the allocation of such diversity.

As has been observed in other plant species, a large proportion of the genetic diversity is found among individuals (within populations). However, some population markers and rare alleles were found in *B. grandiflora* indicating that there is some structure to the genetic composition of this species. The weak genetic structure may be due to a short time having elapsed since the present populations became fragmented or to present gene flow. Given that gene flow estimates were on average less than a migrant/generation, and therefore not enough to prevent genetic drift, it appears that the first hypothesis is more likely.

It was shown that the Sun Ray and Saddleblanket preserves have the populations with the highest diversity. It is plausible that the southern end of the Lake Wales Ridge was a refugium for *B. grandiflora* during one or more glaciations, from which this species recolonized suitable habitats in central and north-central Florida. This hypothesis could be tested by analyzing the genetic structure of other scrub endemics not limited to the Lake Wales Ridge.

Compared to other scrub endemics, *B. grandiflora* appears to have reduced genetic diversity so perhaps bottlenecks during one or more glaciations is not enough to explain this genetic homogeneity since the other scrub species probably suffered the same fate as *B. grandiflora*. Impoverishment of the initial gene pool or strong selection in the harsh environment of the scrub or both may have played a role in the history of this species.

Genetic diversity is reduced in *B. grandiflora* but inbreeding depression does not appear to have great detrimental effects on this species, at least in the early developmental stages if its life cycle. These findings support the idea that genetic diversity is less crucial than it was once thought for the survival of species. Other studies also support this

hypothesis; for example, reduced levels of genetic diversity did not hinder the viability of reintroduced beaver populations (Cheverud *et al.*, 1993). It is also possible that increased diversity is only beneficial during rare events such as new environmental conditions or new diseases or parasites. Perhaps, the amount of genetic diversity required for long term survival is variable for different taxa. The definitive answer is, and probably will remain, unknown. Thus, it is advisable to be conservative when making recommendations for the preservation of any species.

Some population markers and a few private alleles were found, there were also differences in the frequencies of the markers between different populations. Therefore, it would be preferable not to mix the gene pools of the populations unless it is absolutely necessary; for example, if a declining population appears incapable of producing viable seeds. The reason for being so conservative is that once new genes are incorporated into a population it would not possible to remove them if, for example, they caused outbreeding depression.

Outbreeding depression has been reported in another rare plant that grows in fragmented habitats, *Gentianella germanica* (Fischer and Matthies, 1997); in this species crosses with pollen from plants 25 km away from the maternal plant produced seeds with lower germination percentage than crosses from plants 10 m apart. If artificial pollinations with individuals from different regions were to be considered as management tools to increase gene flow, testing for outbreeding depression should be done first.

Because none of the populations surveyed covers the entire genetic diversity range, the largest number of populations and individuals possible should be preserved. At the southern end of the distribution there are several sites where *B. grandiflora* is protected;

The Nature Conservancy Sun Ray, Tiger Creek and Saddleblanket, Preserves and Lake Wales Ridge State Forest in Polk Co.; Wingate Creek State Preserve, Lake Louisa State Preserve and Crooked Creek in Lake Co.; and Flamingo Villas in Highlands Co. (Multi-Species Recovery Plan for the Threatened and Endangered Species of South Florida, Volume I, 1998, Fish and Wildlife Service). In the northern end of *B. grandiflora*'s distribution, the Ocala National Forest in Marion Co. protects a multitude of small subpopulations. There are no protected sites in Orange Co.; conservation of at least one site in this county would be advisable.

B. grandiflora individuals appear unlikely to survive after being transplanted but if it is necessary to relocate adult plants it is probably better to do so during their resting stage, in winter. Mycorrhizae were observed in *B. grandiflora* roots, therefore, to increase the chances of success in propagation attempts soil from the native habitat should be used. Whether the relocation is done by adult plants or seeds, it is advisable to place those individuals in a site where they will not be likely to affect other *B. grandiflora* populations. In case those plants are adapted to local conditions, it would be better to relocate them as close to their original site as possible.

While large preserves might be desirable, there are no large areas that can be placed aside for preservation of *B. grandiflora* or other scrub species. This is especially true in the central area of the geographic distribution in Lake and Orange Co. where development is occurring at a high pace. At the southern end of *B. grandiflora* distribution, the southern Lake Wales Ridge has lost more than 70% of its area to citrus groves and residential development (Peroni and Abrahamson, 1986).

B. grandiflora appears to be present in some scrub islands while it is not found in others that seem equally appropriate for its survival. Other scrub species have also been shown to be absent in suitable locations (Quintana-Ascencio and Menges, 1996); the same observation was made for *Abronia macrocarpa*, an endangered species from Texas (Williamson and Werth, 1999). In *Calamintha ashei*, the presence of seedlings after a fire was related to the presence of adults before such fire (Carrington, 1996). Because of the seed morphology, in all these species long distance dispersal appears unlikely and therefore suitable habitats may remain unoccupied. This lack of good dispersal mechanisms may contribute to the rarity of many scrub species. On the other hand, if the number of protected sites was perceived as too few to adequately protect these rare scrub endemics, perhaps colonization of these seemingly suitable habitats would be a more desirable option than the use of reclaimed areas, although, of course, both could be done simultaneously if enough seeds are available.

Fire has been shown to benefit *B. grandiflora* through increases in the number of above ground stems per plant, increased seedling recruitment, and increase in the percentage of flowers producing seeds (Hartnett and Richardson, 1989). Nevertheless, other types of disturbance may suffice to increase seedling recruitment; the Ocala subpopulation where more than 300 seedlings were observed was not burned. However, this site is regularly disturbed by vehicles and taller plants are suppressed by mowing as this is a power line right-of-way. In the past roadside areas have been dismissed for conservation purposes. However, for many species there are few alternatives. *Grevillea barklyana* road verge populations presented similar or higher genetic diversity and reproductive potential than population in less disturbed areas (Hogbin *et al.*, 1998).

Perhaps roadside areas can be used as corridors to increase gene flow between populations that have been isolated by human intervention.

An important aspect of the biology of *B. grandiflora* that has been little studied is its demography. The above ground portions of the plants dies each winter and does not provide a good estimate of plant age. Large plants probably are relatively old but, depending on the quality of the habitat, it is possible that even small plants can be very old. The growth rate and longevity of *B. grandiflora* are unknown. The underground storage organ of *B. grandiflora* is likely to present the anomalous secondary growth like other members of the Convolvulaceae so determining their age cannot be done by counting rings of xylem. Furthermore, this would be a destructive method and given that *B. grandiflora* in a threatened plant, it would not be appropriate. The remaining option would require the marking of individuals at the stage of seedling and censusing and perhaps measuring their dimensions for several years. This would allow the construction of life tables. The Nature Conservancy personnel has marked *B. grandiflora* individuals and censuses them annually. These efforts indicate that individuals live at least eight years and are likely to persist much longer (D. Gordon personal communication).

Future research should include monitoring the reproductive output of adult plants in established populations to detect decreased reproduction on aging of the individuals or due to changes in environmental conditions. Mortality should also be periodically assessed among previously marked individuals to ensure that there are enough of them to ensure a continued source for recruitment of new individuals. To detect the early effects of inbreeding depression, recruitment should also be monitored.

At present *B. grandiflora* populations appear healthy and stable but they show signs of mild inbreeding depression. If the already low genetic variation is a relict of the past and old individuals are replaced with even less diverse plants, inbreeding could become more critical in the future. Monitoring the extant populations is essential for the perpetuation of this species.

If relocation of *B. grandiflora* plants or recolonization of newly available areas becomes necessary, the genotypes of the individuals used should be taken into account. The crosses of individuals from populations far apart should be tested for outbreeding depression and should be tested in field conditions to detect the lack of adaptation to local conditions. If relocation or recolonization are done, careful records should be kept on the origin of the individuals used and the location of the new populations. Because the consequences of mixing gene pools are unknown, it is preferable to prevent interactions of artificial populations with resident populations unless the latter appear to be in imminent demise.

APPENDIX A
STATISTICAL ANALYSES OF PHENOTYPIC CHARACTERIZATION

Table A-1. Means and standard errors of the logarithm of the number of stems/plant in nine populations of *Bonamia grandiflora* in the Florida scrub.

Populations	Means	Standard errors
Saddleblanket 1	0.41	0.07
Saddleblanket 2	0.89	0.06
Tiger Creek 1	0.28	0.06
Tiger Creek 2	0.27	0.06
Fenton	0.57	0.06
Sun Ray	0.42	0.07
Hull	0.74	0.06
Ocala North	0.61	0.08
Ocala South	0.47	0.07

Table A-2. Means and standard errors of the logarithm of stem length (m) in nine populations of *Bonamia grandiflora* in the Florida scrub.

Populations	Means	Standard errors
Saddleblanket 1	-0.39	0.06
Saddleblanket 2	0.30	0.05
Tiger Creek 1	-0.76	0.07
Tiger Creek 2	-0.70	0.06
Fenton	-0.26	0.07
Sun Ray	-0.18	0.08
Hull	-0.10	0.05
Ocala North	0.05	0.07
Ocala South	-0.09	0.10

Table A-3. Means and standard errors of seed weight (g) in eight populations of *Bonamia grandiflora* in the Florida scrub.

Populations	Means	Standard errors
Ocala	0.0610	0.0012
Creek	0.0680	0.0016
Tower	0.0506	0.0016
Fenton	0.0670	0.0016
Sun Ray	0.0746	0.0009
Hull	0.0648	0.0010
Saddleblanket	0.0668	0.0015
Sugarloaf	0.0550	0.0031

Table A-4. Means and standard errors of seed length (mm) in five populations of *Bonamia grandiflora* in the Florida scrub.

Populations	Means	Standard errors
Tower	6.98	0.06
Fenton	7.23	0.12
Ocala	7.15	0.06
Hull	7.11	0.07
Sun Ray	7.48	0.06

Table A-5. Means and standard errors of seed width (mm) in five populations of *Bonamia grandiflora* in the Florida scrub.

Populations	Means	Standard errors
Tower	4.20	0.08
Fenton	4.59	0.08
Ocala	4.19	0.05
Hull	4.47	0.08
Sun Ray	4.75	0.05

Table A-6. Means and standard errors of the ratio of seed length (mm)/seed width (mm) in five populations of *Bonamia grandiflora* in the Florida scrub.

Populations	Means	Standard errors
Tower	1.67	0.03
Fenton	1.59	0.04
Ocala	1.71	0.03
Hull	1.60	0.03
Sun Ray	1.58	0.02

Table A-7. ANOVA of hair density (number of hairs/mm) in abaxial leaf surfaces in four populations of *Bonamia grandiflora* in the Florida scrub.

Source	df	SS	MS	F	P
Between populations	3	246.68	81.23	1.15	N/S
Within populations	36	2541.10	70.59		
Total	39	2784.78			

Table A-8. Means and standard errors of hair density (number of hairs/mm) in abaxial leaf surfaces in four populations of *Bonamia grandiflora* in the Florida scrub.

Populations	Means	Standard errors
Tiger Creek	36.70	2.69
Ocala	33.70	2.99
Hull	40.40	2.46
Sun Ray	38.50	2.45

Table A-9. ANOVA of hair density (number of hairs/mm) in adaxial leaf surfaces in four populations of *Bonamia grandiflora* in the Florida scrub.

Source	df	SS	MS	F	P
Between populations	3	105.00	35.00	0.41	N/S
Within populations	36	3074.60	85.41		
Total	39	3179.60			

Table A-10. Means and standard errors of hair density (number of hairs/mm) in adaxial leaf surfaces in four populations of *Bonamia grandiflora* in the Florida scrub.

Populations	Means	Standard errors
Tiger Creek	36.90	2.91
Ocala	37.20	3.72
Hull	40.30	2.29
Sun Ray	36.00	2.56

Table A-11. ANOVA of the depth of the cleft in corollas in three populations of *Bonamia grandiflora* in the Florida scrub.

Source	df	SS	MS	F	P
Between populations	2	0.79	0.40	1.61	N/S
Within populations	27	6.61	0.24		
Total	29	7.40			

Table A-12. Means and standard errors of the depth of the cleft in corollas in three populations of *Bonamia grandiflora* in the Florida scrub.

Populations	Means	Standard errors
Fenton	3.37	0.32
Ocala	3.01	0.09
Hull	2.88	0.17

APPENDIX B
STATISTICAL ANALYSES OF REPRODUCTIVE BIOLOGY

Table B-1. Pairwise comparisons of the differences of least square means of the transformed fruiting success index* for six pollination treatments, control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated in *Bonamia grandiflora* (PROC MIXED, SAS). Each cell shows the t-test result (above) and the P-value of the test; df = 221 for all the comparisons the (Satterthwaite approximation).
*Fruiting success index = (#of fruits per plant +1)/(#of aborted fruits per plant + 1)

	Control	Emasc./ Cross-pol.	Emasc./ Self-pol.	Bagged	Emasc./ Bagged
Emasc./ cross-pol.	-0.32 0.752	——	——	——	——
Emasc./ self-pol.	-0.73 0.467	-0.45 0.656	——	——	——
Bagged	6.22 <0.001*	6.99 <0.001*	7.37 <0.001*	——	——
Emasc./ bagged	5.67 <0.001*	6.40 <0.001*	6.76 <0.001*	-0.51 0.612	——
Emasc./ open-pol	1.28 0.2034	1.70 0.0904	2.12 <0.05*	-5.21 <0.001*	-4.64 <0.001*

* Significant differences

Table B-2. Least square means and standard errors of the transformed fruiting success index * for six pollination treatments: control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated in *Bonamia grandiflora* (PROC MIXED, SAS).
*Fruiting success index = (#of fruits per plant +1)/(#of aborted fruits per plant + 1)

Pollination treatment	Least square means	Standard error
Control	0.046	0.043
Emasculated/ cross-pollinated	0.058	0.041
Emasculated/ self-pollinated	0.074	0.042
Bagged	-0.198	0.042
Emaseulated/bagged	-0.178	0.042
Emasculated/ open-pollinated	-0.004	0.042

Table B-3. Means and standard errors of the transformed fruiting success index* for three populations of *Bonamia grandiflora.* (PROC GLM, SAS).
*Fruiting success index = (#of fruits per plant +1)/(#of aborted fruits per plant + 1)

Populations	Means	Standard error
1	-0.042	0.015
2	0.030	0.018
3	-0.098	0.032

Table B-4. Pairwise comparisons of the differences of least square means of the number of seeds per fruit for six pollination treatments, control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated in *Bonamia grandiflora* (PROC MIXED, SAS). Each cell shows the t-test result (above) and the P-value of the test; the degrees of freedom were calculated with a Satterthwaite approximation.

	Control	Emasc./ cross-pol.	Emasc./ self-pol.	Bagged	Emasc./ bagged
Emasc./ cross-pol.	-1.55 0.122	——	——	——	——
Emasc./ self-pol.	0.75 0.453	2.39 <0.05*	——	——	——
Bagged	2.67 <0.01*	3.52 <0.001*	2.33 <0.05*	——	——
Emasc./ bagged	2.27 <0.05*	3.26 <0.005*	1.87 0.063	-0.56 0.577	——
Emasc./ Open-pol.	1.88 0.061	3.43 <0.001*	1.23 0.218	-1.64 0.103	-1.09 0.279

* Significant differences

Table B-5. Least square means and standard errors of the number of seeds per fruit for six pollination treatments: control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated in *Bonamia grandiflora* (PROC MIXED, SAS).

Pollination treatment	Least square means	Standard error
Control	2.756	0.148
Emasculated/cross-pollinated	3.013	0.142
Emasculated/self-pollinated	2.637	0.135
Bagged	1.936	0.292
Emasculated/bagged	2.144	0.254
Emasculated/open-pollinated	2.436	0.149

Table B-6. Means and standard errors of the number of seeds per fruit for three populations of *Bonamia grandiflora.* (PROC GLM, SAS).

Populations	Means	Standard error
1	2.773	0.152
2	2.611	0.156
3	2.740	0.272

Table B-7. Least square means and standard errors of fruit weight for six pollination treatments: control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated in *Bonamia grandiflora* (PROC MIXED, SAS).

Pollination treatment	Least square means	Standard error
Control	0.219	0.011
Emasculated/cross-pollinated	0.217	0.010
Emasculated/self-pollinated	0.201	0.010
Bagged	0.153	0.024
Emasculated/bagged	0.181	0.020
Emasculated/open-pollinated	0.184	0.011

Table B-8. Means and standard errors of fruit weight for three populations of *Bonamia grandiflora.* (PROC GLM, SAS).

Populations	Means	Standard error
1	0.226	0.011
2	0.199	0.012
3	0.186	0.022

Table B-9. Pairwise comparisons of the differences of least square means of the seed weight for six pollination treatments, control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated in *Bonamia grandiflora* (PROC MIXED, SAS). Each cell shows the t-test result (above) and the P-value of the test; df = 221 for all the comparisons (Satterthwaite approximation). * Significant differences

	Control	Emasc./ cross-pol.	Emasc./ self-pol.	Bagged	Emasc./ Bagged
Emasc./ cross-pol.	4.17 <0.001*	——	——	——	——
Emasc./ self-pol.	3.44 <0.001*	-0.82 0.413	——	——	——
Bagged	-3.13 <0.005*	-5.07 <0.001*	-4.66 <0.001*	——	——
Emasc./ Bagged	-2.41 <0.05*	-4.91 <0.001*	-4.48 <0.001*	1.13 0.258	——
Emasc./ Open-pol.	0.62 0.535	-3.30 <0.001*	-2.56 <0.01*	3.41 <0.001*	2.79 <0.01*

Table B-10. Least square means and standard errors of the transformed seed weight* for six pollination treatments: control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated in *Bonamia grandiflora* (PROC MIXED, SAS).
*seed weight$^{2.1}$

Pollination treatment	Least square means	Standard error
Control	0.003123	0.0001816
Emasculated/cross-pollinated	0.002712	0.0001762
Emasculated/self-pollinated	0.002789	0.0001778
Bagged	0.003851	0.0002709
Emasculated/bagged	0.003550	0.0002280
Emasculated/open-pollinated	0.003060	0.0001842

Table B-11. Means of the transformed seed weight* for three populations of *Bonamia grandiflora*. (PROC GLM, SAS).
*seed weight$^{2.1}$

Populations	Means	Standard error
1	0.00325527	0.00009371
2	0.00292123	0.00010591
3	0.00256572	0.00015747

Table B-12. Friedman's test of the percentage of seeds that germinated in six pollination treatments (control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated) and three populations of *Bonamia grandiflora*. The data were ranked by blocks, populations, (PROC RANK) and the sums of squares and mean squares were calculated with GLM for fixed effects; all were done with SAS. Note that because the data were ranked by population, a significant difference among them only reflects a difference in sample size and not a true difference in germination percentage.

Source	df	Type III SS	Type III MS	F	P
Pollination	5	527.138	105.428	1.17	N/S
Populations	2	2321.657	1160.828	12.86	P<0.001
Plants	24	3830.872	159.620	1.77	P<0.05
Capsules	6	442.373	73.729	0.08	N/S
Error	65	5867.489	90.269		

Table B-13. Least square means and standard errors of the ranks of the percentage of seeds germinated for six pollination treatments: control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated in *Bonamia grandiflora* (PROC GLM, SAS).

Pollination treatment	Least square means	Standard error
Control	19.511	4.116
Emasculated/cross-pollinated	19.928	3.542
Emasculated/self-pollinated	15.987	3.299
Bagged	9.816	5.738
Emasculated/bagged	14.596	4.568
Emasculated/open-pollinated	18.024	3.939

Table B-14. Means and standard errors of the percentage of seeds germinated for six pollination treatments: control, emasculated/cross-pollinated, emasculated/self-pollinated, bagged, emasculated/bagged, and emasculated/open-pollinated in *Bonamia grandiflora*.

Pollination treatment	Least square means	Standard error
Control	45.579	2.099
Emasculated/cross-pollinated	52.167	1.691
Emasculated/self-pollinated	38.652	1.408
Bagged	17.851	5.343
Emasculated/bagged	31.636	3.469
Emasculated/open-pollinated	40.737	2.132

Table B-15. Means and standard errors of the percentage of seeds germinated from three populations of *Bonamia grandiflora.* (PROC GLM, SAS).

Populations	Means	Standard error
1	36.957	0.761
2	52.833	1.352
3	35.846	1.502

APPENDIX C
ADDITIONAL DATA AND ANALYSES OF MOLECULAR FINGERPRINTING

Table C-1. Identity of *Bonamia grandiflora* individuals sampled in the RAPD genetic fingerprinting. Individuals were grown in the Ocala National Forest Seed Orchard by the Forest Service personnel from seeds collected in 1991 from several local populations. FR = Forest Route, FT = Forest Trail.

Sample #	Seed lot #	Identity/ population location
FS #13, 22, 34, 43, 51	2	FY 91, Christmas tree area
FS # 3, 38, 42, 44, 50	3	Stand edge on FR 97
FS # 9, 17, 23	7	Sector 32, FR 97-Horse trail
FS # 52, 58	8	100 m E. of FR 97, Sector 29
FS # 35, 40	9	50 m W. of Cor.
FS # 4, 5, 6, 14, 21	10	0.3 mile E. of FR 97 on S-Line
FS # 10, 48	11	0.4 mile N. of S-Line
FS # 53	12	N end of S-55
FS # 37, 60	13	0.1 mile N. of S-Cor
FS # 12, 18	16	79-79 C
FS # 19	19	0.4 mile S. of S-Line
FS # 33, 39, 41, 45, 54	21	0.5 mile S. of S-Line
FS # 1, 7, 15, 20, 47	22	0.25 mile N. of FR 95
FS # 2, 19, 49, 56	24	0.4 mile N. of FT 579-595
FS # 11, 24, 46, 57	25	0.2 mile N. of FT 579-595
FS # 55	26	S.E. Cor. S-3
FS # 16	27	0.5 mile E. of FT 588 on FT 599

Table C-2. Frequency of 46 AFLP bands for six *Bonamia grandiflora* populations. The calculations were performed by POPGENE 1.21 (Yeh, et al., 1997).

Locus	Sun Ray	Saddlebl.	Tiger Cr.	Fenton	Hull	Ocala
1	1.00	1.00	1.00	1.00	1.00	1.00
2	1.00	1.00	1.00	1.00	1.00	1.00
3	0.59	1.00	1.00	0.42	1.00	1.00
4	1.00	1.00	1.00	0.42	1.00	1.00
5	1.00	1.00	1.00	0.42	1.00	1.00
6	0.18	0.12	0.07	0.00	0.00	0.56
7	1.00	0.75	0.75	0.42	1.00	1.00
8	0.18	0.00	0.00	0.00	0.00	0.00
9	0.09	0.06	0.00	0.00	0.00	0.00
10	1.00	0.78	1.00	0.42	0.55	1.00
11	0.09	0.06	0.00	0.18	0.11	0.00
12	1.00	0.75	1.00	0.42	0.55	1.00
13	0.00	0.06	0.00	0.00	0.00	0.00
14	0.09	0.26	0.21	0.00	0.55	0.12
15	0.59	0.57	1.00	1.00	0.11	0.78
16	1.00	1.00	1.00	1.00	1.00	1.00
17	0.18	1.00	1.00	0.00	0.00	0.56
18	0.00	0.13	0.00	0.00	0.00	0.00
19	1.00	0.75	1.00	1.00	1.00	1.00
20	0.42	1.00	1.00	1.00	1.00	1.00
21	1.00	0.53	1.00	1.00	1.00	0.82
22	1.00	0.53	1.00	1.00	0.55	0.56
23	1.00	1.00	1.00	1.00	1.00	1.00
24	1.00	1.00	1.00	1.00	1.00	0.82
25	0.59	1.00	1.00	1.00	1.00	1.00
26	1.00	0.78	0.75	1.00	0.55	0.53
27	0.59	0.78	0.00	0.18	0.37	0.00
28	0.42	0.57	0.64	1.00	1.00	1.00
29	0.00	0.56	0.07	0.18	0.00	0.00
30	0.29	1.00	0.07	0.18	0.11	0.00
31	0.59	1.00	1.00	1.00	1.00	1.00
32	1.00	1.00	1.00	1.00	1.00	1.00
33	1.00	1.00	1.00	0.42	1.00	1.00
34	0.09	0.00	0.00	0.00	0.00	0.00
35	0.59	1.00	0.75	1.00	1.00	0.82
36	0.09	0.00	0.00	0.00	0.00	0.00
37	0.59	1.00	0.75	0.42	1.00	0.74
38	0.09	0.44	0.00	0.18	0.37	0.00
39	0.59	0.26	0.07	0.18	1.00	0.82
40	0.59	0.75	1.00	1.00	1.00	1.00
41	0.09	0.00	0.00	0.00	0.00	0.00
42	0.29	0.06	0.00	1.00	0.23	0.00
43	0.59	0.06	0.00	0.18	0.00	0.05
44	1.00	1.00	0.50	1.00	1.00	1.00
45	0.29	0.20	0.00	0.18	0.37	0.05
46	0.59	0.75	0.75	1.00	1.00	1.00

Table C-3. Homogeneity tests for 46 AFLP bands in six *Bonamia grandiflora* populations. The calculations were performed by POPGENE 1.21 (Yeh, et al., 1997).

Band #	G – test	df	p	Band #	G – test	df	p
1	0.00	9	1.00	24	10.54	9	0.31
2	0.00	9	1.00	25	10.56	9	0.31
3	15.02	9	0.09	26	23.97	9	0.00*
4	10.35	9	0.32	27	28.78	9	0.00*
5	10.35	9	0.32	28	28.14	9	0.00*
6	17.53	9	0.04*	29	28.75	9	0.00*
7	17.83	9	0.04*	30	34.28	9	0.00*
8	4.47	9	0.88	31	10.56	9	0.31
9	2.90	9	0.97	32	0.00	9	1.00
10	17.83	9	0.04*	33	10.35	9	0.32
11	3.69	9	0.93	34	2.07	9	0.99
12	17.34	9	0.04*	35	16.82	9	0.05*
13	2.30	9	0.99	36	2.07	9	0.99
14	10.45	9	0.32	37	21.09	9	0.01*
15	32.67	9	0.00*	38	24.57	9	0.00*
16	0.00	9	1.00	39	28.38	9	0.00*
17	41.08	9	0.00*	40	14.39	9	0.11
18	5.04	9	0.83	41	2.07	9	0.99
19	10.59	9	0.30	42	20.18	9	0.02*
20	15.75	9	0.07	43	14.07	9	0.12
21	17.32	9	0.04*	44	15.33	9	0.08
22	19.24	9	0.02*	45	9.84	9	0.36
23	0.00	9	1.00	46	16.28	9	0.06

Table C-4. Nei's genetic genetic identity (above diagonal) and genetic distance (below diagonal) for 10 populations of *B. grandiflora*. All the calculations were done with POPGENE (Yeh, et al., 1997); Hardy-Weinberg equilibrium was assumed.

	SR	**SB1**	**SB2**	**TC1**	**TC2**	**F**	**H**	**OC 3**	**OC1**	**OC2**
SR	****	0.881	0.879	0.914	0.922	0.899	0.920	0.934	0.898	0.905
SB1	0.126	****	0.888	0.927	0.905	0.866	0.910	0.920	0.895	0.912
SB2	0.130	0.119	****	0.868	0.886	0.809	0.865	0.855	0.850	0.822
TC1	0.090	0.076	0.141	****	0.968	0.897	0.905	0.962	0.937	0.928
TC2	0.081	0.100	0.121	0.033	****	0.882	0.883	0.927	0.914	0.937
F	0.107	0.144	0.212	0.109	0.126	****	0.900	0.895	0.838	0.880
H	0.083	0.095	0.145	0.099	0.124	0.106	****	0.949	0.938	0.920
OC3	0.069	0.084	0.157	0.039	0.076	0.111	0.052	****	0.962	0.955
OC1	0.108	0.111	0.162	0.065	0.090	0.177	0.063	0.038	****	0.921
OC2	0.100	0.092	0.196	0.074	0.065	0.128	0.083	0.047	0.083	****

Table C-5. Geographic distance (km) matrix for 10 populations of *Bonamia grandiflora* in Florida. The distances are the approximated shortest distances between pairs of populations and were measured on maps.

	SR	SB1	SB2	TC1	TC2	F	H	OC 3	OC1	OC2
SR	0									
SB1	8	0								
SB2	8	<1	0							
TC1	23	27	27	0						
TC2	23	27	27	<1	0					
F	85	92	92	68	68	0				
H	90	95	95	72	72	23	0			
OC3	164	169	169	148	148	83	74	0		
OC1	178	183	183	162	162	97	88	10	0	
OC2	171	176	176	155	155	90	81	5	5.5	0

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BIOGRAPHICAL SKETCH

Gabriela B. Romano was born in Argentina. She obtained her Licenciatura en Biología Orientación Ecología at Universidad de la Plata, Facultad de Ciencias Naturales in 1984. She worked in the Venezuelan savannas for two years observing feeding behavior in Black Howler monkeys for the Smithsonian Institution as well as recording the phenology of twenty native species. She moved to the U.S. and entered the graduate program in the Department of Botany, at the University of Florida, under the direction of Francis E. Putz. She took numerous courses and obtained an M.S. in 1990 for her work on dispersal of weed seeds into forest gaps. She worked for several months in Costa Rica, at La Selva under the direction of John J. Ewel. She enrolled as a doctoral student in the Department of Botany, University of Florida, under the direction of David A. Jones.

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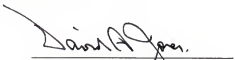

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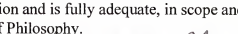

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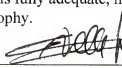

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This dissertation was submitted to the Graduate Faculty of the of the Department of Botany in the College of Liberal Arts and Sciences, and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1999

Dean, Graduate School